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BIOLOGICAL BULLETIN

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The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review by the Board.

1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

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THE

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PARTICLE SORTING AND LABIAL PALP FUNCTION IN THE PACIFIC OYSTER *CRASSOSTREA GIGAS* (THUNBERG, 1795)

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Controversy still attends the question whether bivalves exercise selectivity over ingested material. Various interpretations have been advanced, mainly based upon comparison of stomach contents to the suspension in the environment, and the direct observation of various particles on the exposed pallial surfaces. The functional interpretation of the exquisitely complex ciliation of the pallial organs, particularly by Atkins (1937a, 1937b, 1937c) is predicated upon the movement of particular cilia and tracts and their affect upon single particles. The suggestion is made here that the cilia of the ctenidium and palpi act primarily upon mucus and only indirectly on individual particles. Further, it is suggested that the overall functioning of the labial palpi has been hitherto misunderstood.

Particle sorting activities fall into two phases: the segregation of inorganic particles from nutritious ones, and possible selectivity between various organic entities (*i.e.*, bacteria and phytoplankters). Fox (1936) found that *Mytilus californianus* exercised a marked ability to sort inorganic from organic particles, though some inorganic material did become ingested. According to Coe (1947), up to one-half of the stomach contents in *Tivela stultorum* may consist of sand grains 200–300 μ diameter, but this need not be interpreted as a lack of selectivity because interstitial and periphytic micro-organisms may be the principal dietary source and sand may be used for grinding food, as is most probably the case in the carnivorous septibranchs. Oysters generally do not ingest quantities of large mineral particles, though Nelson (1923) stated that under turbid conditions much sand was present in the stomach of *C. virginica*. It has been suggested that sorting, if it occurs, is merely based upon particle size. Jorgensen (1960) found that *Mytilus edulis* was unable to retain large molecules of haemoglobin, but did filter particles as small as

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1μ diameter. These findings conflict with those of Tammes and Dral (1955) who noted a decreasing ability in mussels to retain the smaller particles.

This paper examines the various possible mechanisms involved in the sorting activities of the Pacific oyster and brings forward evidence as to the function of the labial palpi in that species.

Materials and Methods

Inhalant and exhalant apertures were measured photographically in living subjects, then oysters of various sizes were relaxed in chloral hydrate solution, and the pallial organs dissected and measured. Pumping volumes were monitored at various temperatures using the Galtsoff (1926) constant-level tank with the Moore (1910) rubber apron supplemented by velocity determinations with heated thermistor probes (McCammon, 1965).

The labial palpi were observed in the entire animal by means of an American Optical Pan Endoscope cystoscope inserted through a small channel cut into the shell margin. The speed and direction of mucus flow were monitored by means of colloidal graphite and sand grains upon the excised palpi, and in whole specimens by careful removal of the anterior portion of the shell. Animals in this condition survived several months and appeared to function normally.

A culture of *Chromatium warmingii* Cohn, 1875 purchased from the American Type Culture Collection (No. 14959) was grown in hydrogen sulphide liquid medium with added vitamin and trace elements. *Chromabacterium,* probably *C. amethystinum* Chester, 1897 was isolated from river water and grown on nutritive glucose agar at 25° C for 5 days. For presentation to the oysters, the *Chromatium* medium was centrifuged at low speed for 10 minutes, and the sedimented bacteria resuspended twice with 0.45 μ filtered sea water. Colonies of *Chromabacterium* were removed from the agar surface with a loop and dispersed by brisk agitation in 25‰ sea water. Test oysters were held in shallow trays maintained at 15° C, and supplied with various suspensions of bacteria, either alone, or mixed with the algae *Tetraselma* and *Chlorella.*

Results

The pallial complex may be considered as a simple pump housed in a chamber (inhalant chamber) provided with a restricted inlet (inhalant aperture) and a larger exit (exhalant aperture). The ctenidium functions as a large diaphragm which is also porous to water. Measurements of three representative oysters are given in Table I and the relationship shows schematically in Figure 1. The relative size of the inhalant and exhalant apertures is of great theoretical importance and in *C. gigas* appears to be contrary to the majority of bivalves where the inhalant aperture is substantially larger. In the Pacific oyster the mantle borders are constantly moving so the relationship is not fixed and much of the exhalant region consists of the promyal chamber exit, where water flow may be negligible though the pallial borders are separated.

Noteworthy is the uniformity of ostial size (*ca.* 2900 μ^2), smaller oysters having fewer, rather than smaller, ostia. An individual of 8.2 cm shell length possessed an average inhalant aperture of 0.51 cm^2 and exhalant aperture of 1.42 cm^2. Total

TABLE I

Measurement of ctenidial apparatus for three Pacific oysters

	A	B	C
Shell length (cm)	3.1	8.2	15.0
Meat weight (g)	6.0	27.2	43.9
Ctenidial length (cm)	1.7	6.0	13.0
Ctenidial width	0.5/0.6	1.2/1.5	1.2/1.7
No. of plicae	580	1300	1760
No. of ostia	3.5×10^5	1.85×10^6	2.69×10^6
Area			
Ctenidium (plain) cm^2	9.6	45.6	79.0
Ctenidium (total)	32.6	119.7	174.0
Ostia μ^2 (each)	2900	2900	2900
Total ostia cm^2	10.1	46.6	78.2
Inhalant aperture mm^2	14	51	85
Exhalant	35	142	243
Width of valve opening cm	0.3	0.4	0.6

potential ostial openings amounted to 46.6 cm^2. It is unlikely that all ostia are fully opened simultaneously but if only 50% are functional a much greater total area for water transportation is presented by the openings of the ctenidium than by the inhalant or exhalant areas.

It is well established that the introduction of a partial obstruction in a fluid flow system results in a pressure rise proximal to the obstruction and a fall on the distal side, so water drawn through the relatively small inhalant passages accelerates, then the flow speed is reduced and the ostial openings are probably negotiated at a very low velocity.

Particles settle under the influence of gravity, behaving according to the Stokes equation, and as particles smaller than 200 μ diameter attain terminal velocity almost instantaneously, the constituents of natural suspensions respond quickly to dynamic changes in their environment. It is assumed that to keep a settling particle in suspension, active agitation, or a constant upward flow component in the medium at least equal to the settlement velocity of the particle in question is needed. The theoretical settlement velocities for various-sized particles of 2.6 specific gravity are shown in Figure 2 together with the maximum pallial velocity for a given pumping rate for an 8-cm oyster. The maximum measured and theoretical inhalant pallial velocity is in the order of 18 mm/min. Describing a trajectory through the inhalant pallial chamber, it is unlikely that any particles of high specific gravity larger than 14 μ diameter would impinge upon the ctenidium, but would fall short to the inner mantle surface. So the chances of heavy inorganic particles reaching the ctenidium are much less than for organic particles which nearly approach the specific gravity of sea water.

It may be concluded that the arrangement of the pallial cavity with a relatively small inhalant aperture and a large total ostial aperture, thereby decreasing approach velocity to the ctenidium, is, in fact, a most efficient settlement chamber.

This gravimetric mechanism may be termed the preliminary sorting method, and observations undertaken upon several other bivalve families and genera show that it is probably universal among the ctenidial Bivalvia. It prevents the impingement of mineral particles upon the delicate tissue of the ctenidium and it is probable that the greater proportion of inorganic material rejected in the pseudofeces has not been in contact with either the ctenidia or labial palpi, but settled directly upon the inhalant chamber mantle surfaces.

Those suspended particles, both organic and inorganic, that successfully cross the low velocity zone of the inhalant pallial chamber, either impinge upon the ctenidium or pass through the ostia and are carried away in the exhalant stream. Once on the ctenidium, particles are entrapped in mucus and transported by the activity of the frontal cilia. There appear to be definite thigmotropic sensors upon the ctenidial filaments, as stimulation of a single filament does not appear to increase the mucus flow, but stimulation of two or more adjacent filaments results in a copious secretion. Ctenidial mucus is elaborated by two distinct types of cells. The outer frontal surfaces of the filaments have many columnar eosinophilic cells, while the lateral regions contain nests of wide goblet cells.

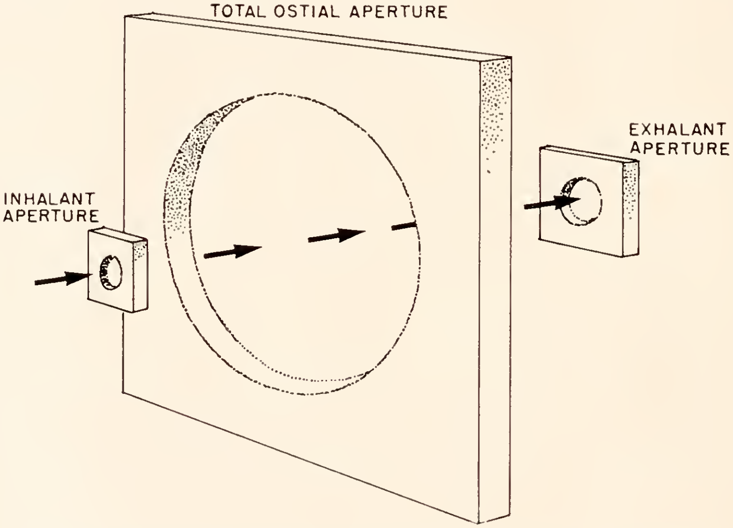

FIGURE 1. Schematic representation of the relative areas of the inhalant, ostial, and exhalant apertures in the Pacific oyster.

The ctenidial surfaces are normally covered with a watery serous fluid, approximately 5 μ thick, which is not subject to ciliary movements, while a definite band of mucus overlying the frontal cilia forms a zone about 12 μ thick and 20 μ wide on each filament. This is a mucus concerned with particle entrapment and is elaborated by the eosinophilic cells. The ctenidia are extremely sensitive to disturbance, either by tactile, temperature, or light stimulation and react in the abundant production of "rejection" mucus which forms a 250–400 μ sheet over the ctenidium and is elaborated by the laterally situated goblet cells. These mucus masses are invariably carried to the free margins. Mucus entering the terminal grooves to reach the mouth must perform a turn of around 180°. The thick layers of mucus are unable to do this and fall off the ctenidium onto the inner mantle margins where they are rejected as pseudofeces; this separation of unwanted mucus is aided by the activity of the lateral groove cilia.

It is highly probable then that two distinct mucus types are concerned with the entrapment and rejection of particles while gravimetric settlement in the inhalant chamber readily accounts for segregation of organic from inorganic particles, such as the report by Allen (1921) noting separation of a blue-green alga from coarser materials.

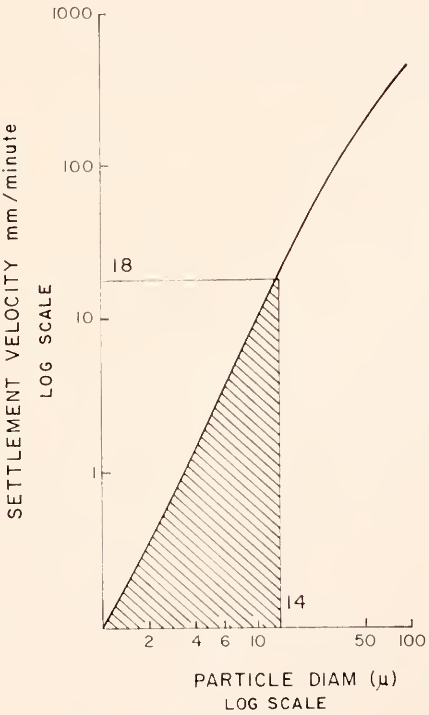

FIGURE 2. Relationship of settling velocity to particle size assuming a specific gravity 2.6 Ordinate 18 is the maximum inhalant pallial velocity for an 8 cm Pacific oyster.

There are a number of reports dealing with separation between various organic forms such as yeasts and algae or algae and bacteria. The central prop to these observations is the report by Loosanoff (1949) that *C. virginica* is able to segregate purple sulphur bacteria from algae. As resolution of this point is central to a better understanding of oyster nutrition, Loosanoff's (1949) experiments were repeated using two genera of chromatophoric bacteria. The autotroph *Chromatium warmingii* is probably identical with the species used by Loosanoff and the other species, a heterotroph, *Chromabacterium cf amethystinum* was also used.

Both bacteria were retained and ingested when presented as pure cultures. If the total load (wet weight) exceeded 200 mg/liter, rejection occurred with production of bright purple pseudofaeces. With lighter loads no rejection was evident and ingestion occurred. If the *Chromatium* was not centrifuged out of the growing medium and washed, substantial to total rejection occurred, probably the result of irritation to the ctenidium by traces of hydrogen sulphide. When mixed with *Tetraselma* ingestion followed, but the bacteria speedily lysolized in the stomach, so that a predominance of algal cells remained. This was more evident with *Chlorella* than *Tetraselma*, the former passing almost undigested through the gut and producing bright green faeces. This clearly demonstrates that the Pacific oyster readily takes up and digests purple sulphur bacteria and only rejects them when their total load exceeds 200 mg/liter.

While ctenidial sorting activities are figured in functional interpretations, the implicated organs have chiefly been the labial palpi. As early as 1851 Alder and Hancock suggested that the palpi of *Pholas* and *Mya* are responsible for active particle sorting. The theory of palp ciliary sorting was much furthered by Kellogg (1915). These studies were made by the observation of the ridged surface and the behavior of small particles placed upon them (Galtsoff 1964), generally with the added insult of brilliant illumination. With this approach, Yonge (1926) found that carmine grains may be transported towards the mouth while carborundum particles are rejected at the edge of the palpi. Menzel (1955) noted similar sorting in *C. virginica* and *O. equestris*. Earlier, Yonge (1923) had put forward the opinion that gravity, causing heavy particles to settle in the interridge grooves,

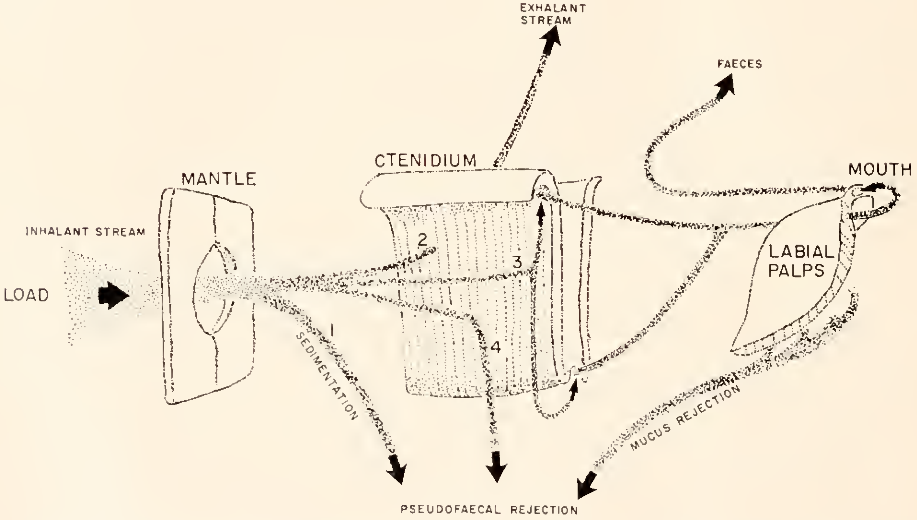

FIGURE 3. Schematic representation of paths and fate of particulate material drawn into the inhalant pallial cavity; (1.) sedimentation of particles of high specific gravity; (2.) passage through the ostium; (3.) impingement upon ctenidium and transportation on frontal mucus bands to food grooves; (4.) rejection of large mucus masses.

resulted in sorting. This is entirely untenable, as at the best only two surfaces can be suitably positioned to take advantage of gravitational separation; furthermore, observations of many different bivalve taxa reveal that in their natural positions the rejecta of the palpi move in an upwards direction.

Observation of the labial palpi by means of a cystoscope shows that in *C. gigas* and other species the two palp lamellae are always closely appressed. When considered as an entity, rather than two separate surfaces, the result of ciliary activity is different. Nelson (1923) found that when narcotized with magnesium sulphate the ridges of the palpi of the oyster collapsed, resulting in large quantities of mucus and food blocking the buccal cavity, and Churchill and Lewis (1924), working on the freshwater *Anodonta,* concluded that the palpi functioned to reduce the quantity of material entering the mouth, but did not discuss the basis for their assertion.

During the course of many hundreds of dissections it was noted that though the food grooves and ctenidia bore light-colored and almost transparent mucus, the mucus of the buccal cavity and stomach was dark brown and contained many more particles.

Table II

Dry and wet weights of food groove and buccal mucus from Pacific oysters

	Food groove			Buccal cavity		
	Wet	Dry	% solid	Wet	Dry	% solid
	0.2967	0.0122	4.1	0.1873	0.0237	12.6
	0.3456	0.0177	5.1	0.8380	0.0691	8.2
	0.2536	0.0093	3.6	0.3372	0.0334	9.9
	0.4611	0.0064	1.3	0.5611	0.0592	10.5
	0.3371	0.0082	2.4	0.3825	0.0506	13.2
	0.5200	0.0193	3.7	0.6730	0.1082	16.0
	0.4766	0.0098	2.0	0.4201	0.0566	13.5
$\bar{X}$	0.3843	0.0118	3.1	0.4856	0.0572	12.0

The dissection of actively feeding oysters and rapid removal with a micropipette of samples of the food groove mucus and buccal mucus and their wet and dried weight differential revealed that the ctenidial mucus has an average 3.1% dry weight, but the buccal mucus 12.0%, a fourfold increase in particulate matter. The data for seven oysters are given in Table II.

During water pumping, whether food particles are present or not, a continuous stream of mucus is moving towards the mouth from the ctenidial region. It was established that an individual oyster with an 8.2 cm total shell length has a ctenidial area of approximately 120 cm^2, with 50 cm^2 of this consisting of the mucus bands overlying the frontal cilia. In the undisturbed state at 20° C this mucus travels approximately 131 mm/min. This layer is usually 12 μ in thickness so that it may be calculated that 7.9 cm^3 of mucus would enter the mouth each hour. This is a substantial volume when the stomach capacity is approximately 0.75 cm^3.

Discussion

It is well established that bivalve stomach contents are not representative of the particles suspended in the environment, but there is a degree of selectivity in the particles retained, either a subsequent sorting activity by the pallial organs or the result of non-retention. Other particles, those of greater or lesser specific gravity than sea water, will describe trajectories through the low-velocity zone of the inhalant pallial cavity and are unlikely to impinge upon the ctenidium. Those particles that are retained on the ctenidium are suspended in the frontal mucus bands and carried towards the food grooves. Overstimulation of the filaments by large masses of food or large particles will cause the release of the heavier rejectory mucus which is removed from the ctenidium either by muscular action or by its inability to enter the food grooves. This is the mucus comprising the 'mucus-net' of MacGinitie (1941) and does not represent the normal pumping-feeding configuration. The subjects were either reacting to unfavourable water conditions or microscopic examination was protracted and the ctenidia disturbed by high levels of illumination. Further evidence of their distressed condition is evidenced by the activity of the labial palpi reported by MacGinitie. In the normal feeding situation the palps are appressed, moving only to detach rejection mucus from their free edges.

An explanation of Loosanoff's (1949) observations may lie in the digestive ability of the oyster for bacteria. Both *Chromatium* and *Chromabacterium* placed in filtered stomach juice undergo lysolysis in 6 to 10 minutes. It may be speculated that while both algae and bacteria were being simultaneously ingested, the preferential digestion of bacteria resulted in bacteria-free faeces. The colored pseudofeces show that most of the suspension was being rejected due to the high concentration of H_2S undoubtedly present if *Chromatium* was proliferating. If the bacterial population was sufficiently high the purple colouration would mask the green of the algae, resulting in purple pseudofeces, while the digestive activity of the gut would produce brownish-green faeces. This interpretation is supported by the rapid microscopic examination of gut contents where many bacteria were found.

The literature is almost unanimous in interpreting palp function as sorting unwanted particles prior to ingestion. This is based entirely upon the study of mucus movements over isolated palp surfaces, yet in the normal state the inner surfaces of the palpi are always pressed together resulting in a "sandwich" of mucus between opposing ciliated surfaces. The mucus-bearing food particles are carried completely within the oral groove, so are not affected by the ciliary activity upon the palp ridges. However, the depths of the interfolds bear ciliary tracts that beat away from the central food groove, so there is a trend to remove mucus from the groove axis. If the mucus is free-flowing it will eventually be carried to the palp margin and discarded. If particulate matter is included, the mucus will come under the influence of the other ridge ciliary tracts and be carried to the crests of the folds where it will be transported in an anterior direction parallel to the palp length. A band of cilia on the posterior upper surface of each fold beats in the opposite direction. The net result of this complicated ciliation is to bring any large mucus masses to the free edge of the palp for rejection. Fine mucus is drawn off by the outward-beating cilia at the bottom of the folds and particulate matter is carried over the folds and returned to the food groove.

Ridewood (1903), commenting upon genera with very different ctenidial structure, noted the striking similarity of the labial palpi characterized in most taxa by transverse ciliated ridges borne on two opposed surfaces, the sole exception being in *Solemya, Huxleyea,* and the closely related *Nucinella.* In macrophagous forms the palpi are reduced and may be entirely absent, as in the carnivorous septibranch *Cuspidaria,* or reduced to unciliated flaps in *Poromya.* This would indicate the universality of the functioning of these organs in the filter- and deposit-feeding Bivalvia. The particle-concentrating capability of the Pacific oyster palpi is approximately fourfold. Under similar feeding conditions to produce 1 g dry weight material requires 32.6 g of food-groove mucus while only 8.5 g buccal mucus. While the oyster palpi are predominantly mucus reducers they possess the ability to reject their entire load. In a normal functioning state they are covered with a serous mucus similar in consistency to the ctenidial frontal filament mucus, but the subepidermal goblet cells secrete copious quantities of rejectory mucus when stimulated.

Bivalves, encased in their shell envelope, lack external food-gathering organs, with the possible exception of the protobranch palp proboscides and, in some taxa, the foot. They have developed a complex mechanism for bringing food, suspended in the inhalant stream, into the pallial cavity, retaining a portion of these and conveying them to the mouth. The major sorting activity is the segregation and rejection of heavy mineral particles, accomplished due to the architecture of the pallial complex, rather than an intrinsic physical sorting mechanism of the pallial organs. I conclude that ctenidial selectivity is limited to a simple acceptance or rejection of particles and mucus present on a particular lamellar segment, while the major function of the oral palpi is as reducers of mucus volume prior to ingestion. The integrated mechanism proposed for the Pacific oyster is represented schematically in Figure 3.

SUMMARY

1. The pallium of the Pacific oyster comprises a restricted entrance and exit to a chamber separated by the ctenidium, analogous to a large diaphragm pump. This configuration results in a low water velocity in the inhalant cavity.

2. Separation and rejection of particles of high specific gravity occurs prior to impact upon the ctenidium, due to gravitational settlement because of the low pallial water velocity.

3. Particles settling on the ctenidium are either rejected or carried in mucus to the labial palpi where the volume of mucus is reduced, and the concentrated food-mucus mass passed to the mouth.

4. Pacific oysters are not able to sort purple bacteria from unicellular algae, but rapid digestion results in bacteria-free alimentary canal and faeces.

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PHOTOPERIODIC CONTROL OF DEVELOPMENT IN *CHAOBORUS AMERICANUS* WITH SPECIAL REFERENCE TO PHOTOPERIODIC ACTION SPECTRA

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Despite the profusion of literature on photoperiodism of insects and related forms, there are relatively few reports which provide energy-compensated action spectra (Lees, 1953, 1966, 1971; Norris, Howell, Hayes, Adler, Sullivan, and Schechter, 1969; Bradshaw, 1972). Experiments concerned with the action spectra of photoperiodism in insects may be divided into three categories. The first involves the use of light sources and/or distribution of various wavelengths without regard to intensity (Kogure, 1933; Geyspitz, 1957; Williams, Adkisson, and Walcott, 1965). The second category of experiment establishes action spectra by equalizing energy or flux density of incident photons (de Wilde and Bonga, 1958; Müller, 1964; Harris, Lloyd, Lane, and Burt, 1969). If one is dealing with the appropriate energy or photon density level, this type of experiment is very useful. Often the energy value chosen is too high. Thus, Müller (1964) chose 160 ergs cm^{-2} sec^{-1} and found that the homopteran, *Euscelis,* responded to an entire series of wavelengths from violet to orange; presumably, many of these wavelengths would have been ineffective at lower intensities. The third category of experiments includes the action spectra where energy of flux density and wavelength are varied to give a standard response. It is this type of experiment which gives the most information. Indeed, once one knows the photon efficiency spectrum, then an equal flux density spectrum can be used effectively to monitor changes in sensitivity.

A suitable insect for action spectrum studies is the larva of the non-biting mosquito, *Chaoborus americanus.* Apart from their eyes and hydrostatic organs, the larvae are devoid of pigment and are available in the huge quantities necessary. The overwintering larvae rely upon photoperiodic and trophic signals for the maintenance or termination of diapause (Bradshaw, 1969). Ordinarily, food and long days must be present simultaneously and not sequentially to elicit development. The larvae are polymorphic and in some years, long days alone suffice to terminate diapause among the larger, yellower larvae (Bradshaw, 1973). In any year after prolonged chilling, the larger, yellower larvae will develop if fed on short-day photoperiod. Food contributes to post-diapause survivorship but for the termination of diapause *per se,* feeding constitutes an environmental cue independently of nutrition (Bradshaw, 1970). The present investigation considers responsiveness of diapausing larvae to photoperiod, the spectral sensitivity of this responsiveness, and the transition from photoperiod-dependent to photoperiod-independent development.

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MATERIALS AND METHODS

Large numbers of larvae were collected from beneath the ice in George Pond, a small eutrophic kettle hole on the University of Michigan's E. S. George Reserve, near Pinckney, Michigan. The larvae were transferred on the day of capture to open jars and the latter stored in 5° C incubators programmed to provide 8 hours of light per day. No food was added. For experimentation, the larvae were warmed to room temperature and the largest yellow larvae sorted from the rest. The large yellow larvae are the fastest responding morph in the wild population (Bradshaw, 1973) and provided the most uniform results in experiments of the sort presented here. Ordinarily, larvae were exposed to an experimental regimen for only a few days and then placed under short-day conditions without food for an additional 5 to 10 days. The reason for using only a few days exposure to the test regimen lies in the need to conduct experiments during the annual period when the diapausing larvae are most responsive to photoperiod. Further prolongation of each experiment would not have permitted determining the response of the animals over the required range of intensities and wavelengths. Food in all cases consisted of mosquito larvae, *Culex pipiens*, provided in saturating amounts each day. For reasons given earlier (Bradshaw, 1969), the appearance of pupae was used to score the termination of diapause and resumption of development.

EXPERIMENTAL RESULTS

Responsiveness of larvae to photoperiod

The following experiments are intended to assess the range of photophases affecting development. Sample populations of diapausing larvae were provided 12–17 hours of light per day in one hour increments at 23 ± 1½° C. Fifty larvae collected January 20, 1968, were exposed to each test daylength with food for

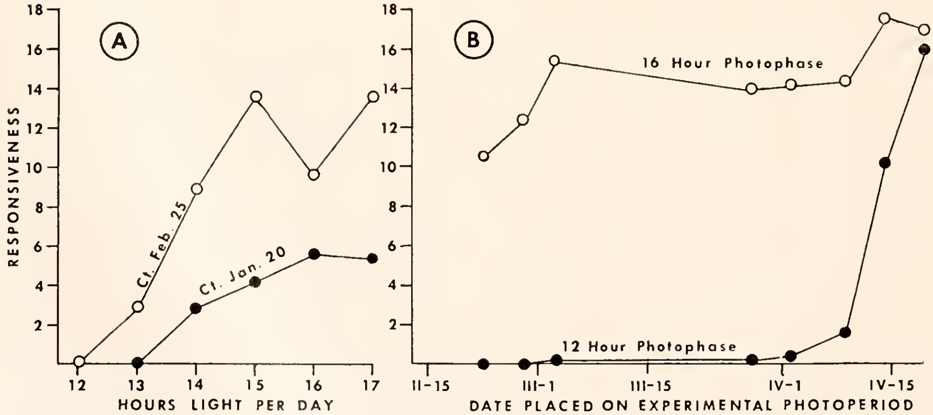

FIGURE 1. Responsiveness of diapausing larvae to photoperiod. (1A) Responsiveness (per cent pupation per day of exposure to experimental regimen) of larvae caught January 20 and February 25, 1968, and exposed to various photophases April 12 and April 22, 1968, respectively. (1B) Responsiveness of larvae caught February 13, 1969, stored under short-day conditions at 5°C, and exposed to long- or short-day photoperiods on various dates in 1969.

seven days, starting April 12, 1968. Fifty larvae collected February 25, 1968, were exposed to each test daylength with food for five days starting April 22, 1968. After seven and five days, respectively, the food was removed and the larvae placed on a 12L: 12D regimen at 23 ± $1\frac{1}{2}$° C for a total experimental time of 15 days. Development was then scored and the experiment terminated. Responsiveness of larvae was calculated as per cent pupation divided by the number of days exposure to the test daylength.

Figure 1A shows that responsiveness generally increased with longer photophases. At any given daylength, however, the larvae collected earlier in the year were less responsive than those collected at a later date.

Changes in responsiveness during the year were further examined with respect to long- and short-day photoperiods, as defined by the above experiments. Diapausing larvae caught February 11, 1969, were periodically removed from the stock incubator from February 22, to April 17, 1969. One hundred larvae were exposed to either 12 or 16 hours of light per day with food for 5 days at 23 ± $1\frac{1}{2}$° C. The food was then removed and both sets of larvae placed on a 12L: 12D regimen for 10 additional days at the same temperature. After a total experimental time of 15 days, development was scored and the experiment terminated. Responsiveness was calculated as above.

Figure 1B shows that responsiveness to both long and short days with food increased during the year. The change in responsiveness to long-day photoperiod was gradual; that to short-day photoperiod was abrupt. Responsiveness to short days increased tenfold over a 10 day period.

Action spectra

The following experiments test the effectiveness of various wavelengths in provoking the termination of diapause. Diapausing larvae were exposed to a short day of white light (12L: 12D) onto which was added four hours of monochromatic light (Bradshaw, 1972). If the larvae "saw" the colored light, the regimen was interpreted as long-day and pupation ensued; otherwise, the larvae would remain in diapause. For each experiment at each wavelength studied, 50 diapausing larvae were exposed to the test regimen for 5 or 7 days with food at 23 ± $1\frac{1}{2}$° C. The food was then removed and the larvae placed under a short-day regimen at the same temperature. After 5 additional days, development was scored and the experiment terminated. Duplicate long-day (16L: 8D) and short-day (12L: 12D) controls were run in parallel with every experiment. For each wavelength studied, intensity of incident light was varied in equal energy logarithmic increments until the response to monochromatic light was no longer significantly different from the long-day, white-light control (normal variate not significant at the 5% level of confidence).

To allow for changes in responsiveness, error introduced in sorting the animals, and fluctuations in temperature, the raw percentage data were transformed to a standardized percentage value by the equation:

$$\text{Standardized \% Response} = \frac{(\text{raw experimental \%}) - (\text{\% response of short-day control})}{(\text{\% response of long-day control}) - (\text{\% response of short-day control})}$$

A dose-response curve was then calculated for each wavelength using these standardized percentage values. Action spectra were finally constructed for the threshold (arbitrarily, the 10% intercept), 50%, and saturation (arbitrarily, the 90% intercept) responses in the dawn and in the dusk.

Both at dawn and at dusk, diapausing larvae were most sensitive to light at 540 nm in the yellow-green region of the spectrum (Figure 2). For a saturating response, larvae were about two orders of magnitude more sensitive to light at 540 nm in the dawn than in the dusk. The shape of the known portions of the spectrum for these responses is similar to that of saturation, namely, around 540 nm. The action spectra demonstrate that overwintering larvae are extremely sensitive to small quantities of light at 540 nm, probably less than 10^{-2} ergs cm^{-2} sec^{-1}.

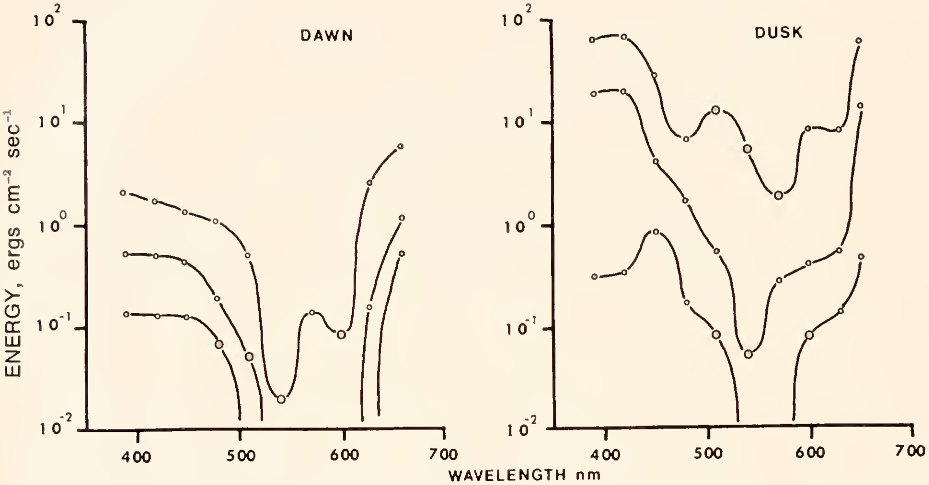

FIGURE 2. Action spectra for photoperiodic induction of development in response to dawn and dusk pulses of monochromatic light. Energy necessary to promote a threshold (bottom curve), 50% (middle curve), or saturation (top curve) response is plotted as a function of wavelength; open circles, interpolated point; dotted circles, extrapolated point.

Sensitivity to light during the dark period

Sensitivity to light during the dark period was assessed by providing asymmetric skeleton photoperiods. Samples of 50 larvae without food were exposed to eight hours of white light plus a 30 minute white-light pulse at various times during the dark period. Thus, successive samples would receive 8L : $\frac{1}{2}$D : $\frac{1}{2}$L : 15D, 8L : 1D : $\frac{1}{2}$L : 14$\frac{1}{2}$D, . . . 8L : 15D : $\frac{1}{2}$L : $\frac{1}{2}$D. Two control experiments of 100 larvae each were run concurrently and consisted of a 17L : 17D regimen without food. Larvae experienced asymmetric skeleton or control photoperiods for 10 days at 23± 1$\frac{1}{2}$° C after which they were transferred to short-day photoperiod (12L : 12D) at the same temperature. Development was scored after 5 additional days and the experiment terminated.

As shown in Figure 3, 30 minute light breaks were most effective at two times during the dark period. Seventy-six and 71 per cent of the larvae were stimulated to pupate by light pulses ending 14½ and 16½ hours after dawn, respectively. A higher percentage of larvae were stimulated to develop by 17 hours of continuous illumination per day than by any asymmetric skeleton photoperiod.

DISCUSSION

Previous data (Bradshaw, 1969) indicated only that the transition from photoperiod-dependent to photoperiod-independent development in *C. americanus* occurred between February and Late April. The results in Figure 1B show that the transition took place over a 10 day period in early April, 1969. Bradshaw (1969) proposed that the adaptive significance of the shift in photoperiod dependency relates to the shallow pond habitat of *C. americanus*. A thaw accompanied by a plankton bloom may take place in January or February. Refreezing of the pond can follow such a thaw through April. Major reliance upon photoperiodic cues during the winter thus insures against development while the pond is likely to refreeze; when an unfrozen pond is likely to persist, increased responsiveness to trophic cues ensures that *C. americanus* will be able to capitalize on the large spring plankton blooms.

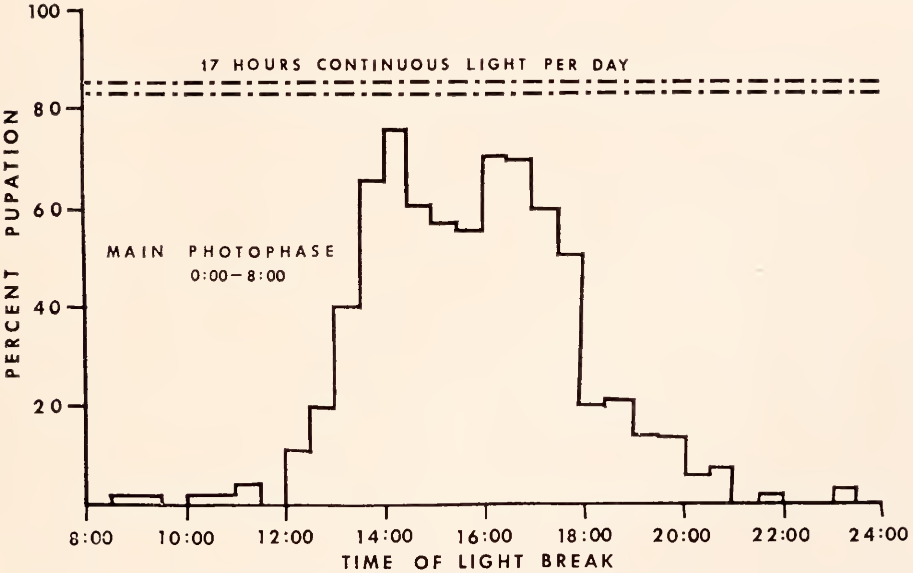

FIGURE 3. Development in response to half-hour light breaks during a 16 hour dark period of an otherwise short-day regimen (8L:16D). The broken lines at the top of the figure represent the response of two control populations exposed to continual long days (17L:7D).

Apart from affecting the qualitative shift in cues relied upon for development, chilling has a more quantitative effect on photoperiodism. As seen in Figure 1A, chilling enhances responsiveness to long and intermediate photophases. The im-

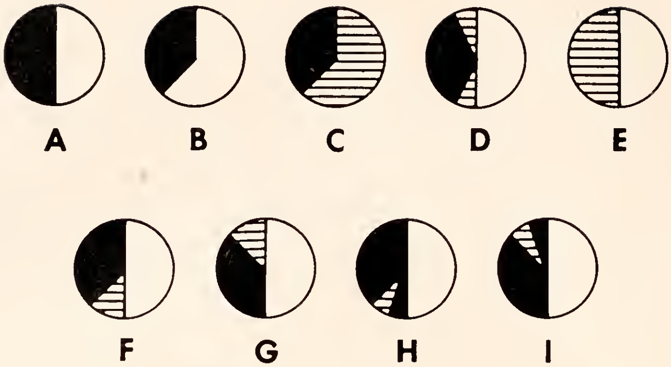

FIGURE 4. Experimental designs for determination of action spectra. Black indicates dark period; shaded, experimental monochromatic light; open, white light; A, hypothetical short day, no response; B, hypothetical long day, full response; C, action spectrum determined with a monochromatic long day in conjunction with a short night; D, action spectrum determined with both dawn and dusk extentions of a white-light short day; E, action spectrum determined with a monochromatic "night" and a white-light short day; F, G, action spectra determined with monochromatic extentions of a white-light short day into long day in the dusk and dawn, respectively; H, I, action spectra determined with monochromatic light breaks at the sensitive period early and late in the subjective night, respectively.

portant implication is that both the critical photoperiod and the number of days required for the termination of diapause are decreasing during the winter and early spring. At the same time, environmental daylengths are rapidly increasing. The net result is to endow the intrinsic accuracy of the photoperiodic clock with increased precision. Consequently, the transition from diapause-maintaining to diapause-terminating daylengths should take place more rapidly under natural conditions than is implied by response to static daylengths in the laboratory.

Given a photoperiodic system, there are many ways to set up an energy-compensated action spectrum (Fig. 4). Each of these methods has its own limitations. If one assumes that a short-day response is evoked by a regimen such as 4A and a long-day response by 4B, then an action spectrum could be determined by substituting monochromatic light for white light (4C), for dark (4E), or by adding short bursts of monochromatic light to both ends of a white-light, short-day regimen (4D). These three methods have the drawback that they only assay for wavelengths and intensities common to both dawn and dusk. Both peak and level of sensitivity could easily change during the day and would not be revealed by a spectrum derived by methods 4C–4E. Furthermore, 4C and 4E do not give a long-day, short-day alternative but rather constant dark *vs* long days (4C) or constant light *vs* short days (4E). Two separate spectra are necessary: one at either end of the white-light photophase. Such spectra would be obtained by regimens 4F and 4G. Alternatively, since light breaks during the dark period of an otherwise short-day regimen (asymmetric skeleton photoperiods) are capable of mimicking an entire photophase (Bünning and Joerrens, 1960; Adkisson, 1963; Pittendrigh and Minis, 1964), comparable spectra could conceivably be obtained by the regimens 4H and 4I. Barker, Cohen, and Mayer (1964) and Lees (1966, 1971) have used this concept. Unfortunately, action spectra defined by light breaks as in 4H and 4I have several limitations. First, the periods of sensitivity must be well defined to make certain one is providing light during the sensitive period; secondly, a whole family of action spectra must be constructed to account for time-intensity reciprocity as observed by Lees (1966, 1971); and thirdly, the period of time between lights-off and the pulse of monochromatic light in the early subjective night may allow the receptor pigment system to dark-adapt. Consequently, the spectra in the present study were determined by the methods in Figure 4F and 4G.

The spectra in Figure 2 show that the photoperiodic clock in *C. americanus* is most responsive to 540 nm light both during the dawn and during the dusk. No other energy-compensated spectra have been determined for other insects using dawn extentions of monochromatic light. There are other energy-compensated spectra using dusk extentions which show that insects may generally have a peak sensitivity in the blue region of the spectrum (Norris, *et al.*, 1969; Harris, *et al.*, 1969). The reason why peak sensitivity in *C. americanus* does not lie further towards the blue probably relates to the larval habitat—characteristically small, shallow Nearctic ponds. In the spring, summer, and early fall, the smallest of these ponds may become covered by the broad-leaved canopy. Larger ponds are usually subject to heavy growths of the green algae, *Nitella* and *Chara*, as well as dense overgrowths of duckweed, *Lemna*. The peak sensitivity of *C. americanus* at 540 nm thus lies where the overlying chlorophylls, carotenes, and xanthophylls are absorbing at a minimum (Rabinowitch and Govindjee, 1969, pages 106–107).

Different action spectra for dawn and dusk would not distinguish between separate pigment systems and distinct photoisomers of the same system. In the case of *C. americanus*, the similarity of action spectra at dawn and at dusk argues that similar or identical photoisomers of a single pigment complex underlie both the dawn and dusk responses. The data presented here are, however, not sufficient to speculate on the type of pigment involved.

The increase in sensitivity at dawn (Fig. 2) could be the result of either an endogenous sensitivity rhythm in the receptor pigment system or light-adaptation during the day. The response of *C. americanus* to asymmetric skeleton photoperiods (Fig. 3) provides the basis for resolving these two possibilities. If the larvae were intrinsically more sensitive to light in the dawn, light breaks in the late subjective night would have elicited more development than those in the early subjective night. The results in Figure 3 show that sensitivity in the late subjective night is no different from or lower than that in the early subjective night. The peak in response at 16½ hours is lower than that at 14½ hours and there is less area under the curve between midnight and dawn than there is between dusk and midnight. The simplest explanation is that of a single pigment system in which the light-adapted pigment is less responsive to light than the dark-adapted one. In Figure 3, the 6 hours of light intervening between lights-off and the first peak in responsiveness permits the pigment to dark adapt. Responsiveness in the early subjective night is then equal to that in the late subjective night. Winfree (1972) has observed a slow dark-adaptation in the resetting of the ecdysis clock in *Drosophila* after transferring pupae from constant light to constant dark. In the case of *Drosophila*, response to phase shifting stimuli required about three days to dark-adapt by an order of magnitude. Though the response of *C. americanus* suggests a faster and greater degree of dark-adaptation, the idea of dark-adaptation by a biochronometric system is not novel. One wonders if dark-adaptation plays a predominate role in determining the sensitivities of photoperiodic mechanisms under natural conditions. If so, action spectra of interrupted nights as performed by Lees (1966, 1971) may have major implications on the physiology of photoperiodism but the extended day technique used in the present study will be more valuable in gaining insight into the ecology of photoperiodism.

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SUMMARY

1. Responsiveness of diapausing *Chaoborus americanus* larvae to photoperiod increases with chilling. Both the critical photoperiod and the number of long or intermediate days required to terminate diapause decrease at the time of year when environmental photophases are rapidly increasing. Consequently, the transition from diapause-maintaining to diapause-terminating daylengths probably takes place faster in nature than is indicated by responsiveness to static photoperiods in the laboratory.

2. After prolonged chilling, short days no longer maintain diapause and food alone suffices to evoke development. The switch from photoperiod-dependent to photoperiod-independent development takes place over only a ten day period.

3. Energies of monochromatic light from 390 to 660 nm required to elicit 10%, 50%, and 90% development were determined by extending an otherwise white-light, short-day regimen to long-day with monochromatic light at dawn or at dusk. Monochromatic light at 540 nm was most effective at evoking development both during dawn and dusk but larvae were about two orders of magnitude more sensitive during the dawn.

4. Response to half hour light breaks during an otherwise short-day regimen suggests that the relative insensitivity at dusk is due to light-adaptation of the receptor pigment system during the white-light day. Greater insight into the ecology of photoperiodism will therefore probably be derived from action spectra based on the extended day technique than from those based on light breaks during the dark period.

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COELOMIC FLUID VOLUME REGULATION AND ISOSMOTIC INTRACELLULAR REGULATION BY *LUIDIA CLATHRATA* (ECHINODERMATA: ASTEROIDEA) IN RESPONSE TO HYPOSMOTIC STRESS

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Despite an inherent inability to control the osmotic pressure of their body fluids, many echinoderms are able to tolerate hyposmotic environments (Binyon, 1966, 1972a). Among asteroids, euryhalinity has been demonstrated and studied in detail only in the genus *Asterias* (Meyer, 1935; Maloeuf, 1937; Smith, 1940; Loosanoff, 1945; Bock and Schlieper, 1953; Kowalski, 1955; Schlieper, 1957, 1958; Binyon, 1961, 1962, 1972b; Jeuniaux, Bricteaux-Grégoire and Florkin, 1962). Reproductively active populations of *Asterias rubens* are found in the Baltic Sea in salinities ranging from 15–17‰ (Kowalski, 1955). Reproductively active populations of the evolutionary primitive platyasterid asteroid, *Luidia clathrata* (Say), similarly are found in Tampa Bay, Florida (Lawrence, 1973). Salinities in the bay range from 18–27‰ as a result of seasonal changes in rainfall. As a consequence of the low and fluctuating salinity levels, this asteroid must be able to tolerate or compensate for large variations in the osmotic pressure of its coelomic fluid.

Preliminary observations indicated that specimens of *Luidia* were able to survive salinities as low as 14‰. Sudden exposure to this low salinity resulted in the cessation of podia activity and overall swelling of the body as has been reported for *Asterias rubens* subjected to hyposmotic stress (Binyon, 1961, 1972b; Jeuniaux *et al.,* 1962). Recovery of activity by these hyposmotically stressed specimens of *Luidia* indicated that compensatory processes were occurring. Florkin and Schoffeniels (1969) have suggested that isosmotic intracellular regulation occurs when extracellular anisosmotic regulation is not present in euryhaline invertebrates. Changes in the intracellular levels of free amino acids associated with isosmotic intracellular regulation have been reported for the echinoid, *Strongylocentrotus droebachiensis* (Lange, 1964) and the asteroid, *A. rubens* (Jeuniaux *et al.*, 1962; Binyon, 1972b).

The purpose of the present study was to ascertain if coelomic fluid volume regulation and intracellular regulation occur in hyposmotically stressed specimens of *Luidia clathrata*, and to associate their time courses with that of the recovery of activity of the intact animal. In addition, the time course of the rate of ammonia excretion of hyposmotically stressed animals was measured to observe if it could be correlated with that of expected changes in the levels of ninhydrin positive substances in the body tissues.

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MATERIALS AND METHODS

Experimental animals

Animals were collected in Tampa Bay, Florida in April, 1973. Ambient temperature was 24° C and ambient salinity was 27‰. The animals were maintained without food in the laboratory in well-aerated, continuously filtered aquaria. Thirty animals each were placed in hyposmotic artificial sea water (Seven Seas Mix; 16‰) and ambient sea water (27‰). Five animals from each group were removed after 1, 2, 4, 6, 8 and 10 days for analysis.

Whole animal analyses

The volumes of the whole animals were determined by measuring the amount of sea water they displaced. By marking the epidermis with nile blue, it was possible to determine the volume of each individual animal at the beginning of the experiment and again when each was used for analysis. From this, the volumes of the animals through the experimental period were calculated as the per cent of its original volume.

The activities of the whole animals were measured by utilizing the activity coefficient (1000/righting time in seconds) as used by Percy (1971) for echinoids. The righting of inverted animals is a basic reflex of echinoderms which involves considerable neuromuscular coordination (Reese, 1966) and should reveal the functional well-being of the animal. Following Percy, the mean activity coefficient ± a standard deviation was used when all individuals in an experimental group righted within a set period of time (10 minutes), and the median activity coefficient was used when not all individuals righted within that period.

Tissue analyses

After the measurements were made on the intact animals, the podia and pyloric caeca were removed from each animal. The podia were scraped from the ambulacral grooves with a spatula and blotted with filter paper to absorb the ambulacral fluid. The pyloric caeca were dissected from the body cavity. Portions of each tissue were weighed, dried in a vacuum desiccator over concentrated sulfuric acid, and reweighed to obtain the per cent hydration.

Ninhydrin positive substances (NPS) were measured by the method of Cocking and Yemm (1954) on tissue extracts prepared with 5% TCA. Alanine was used as the standard. Protein was measured by the method of Lowry, Rosebrough, Farr and Randall (1951) on extracts prepared with 1 N NaOH. Bovine serum albumin was used as the standard. The concentractions of NPS were calculated in terms of g tissue water.

Ammonia excretion

Rates of ammonia excretion were ascertained on animals different than those used above. Five animals each were placed in hyposomotic (16‰) and ambient (27‰) sea water. After 2, 6, 12, 26, and 52 hours, the animals were placed in individual bowls which contained 100 ml of sea water of the same concentration as that from which they had been taken. At the end of 2 hours, water samples

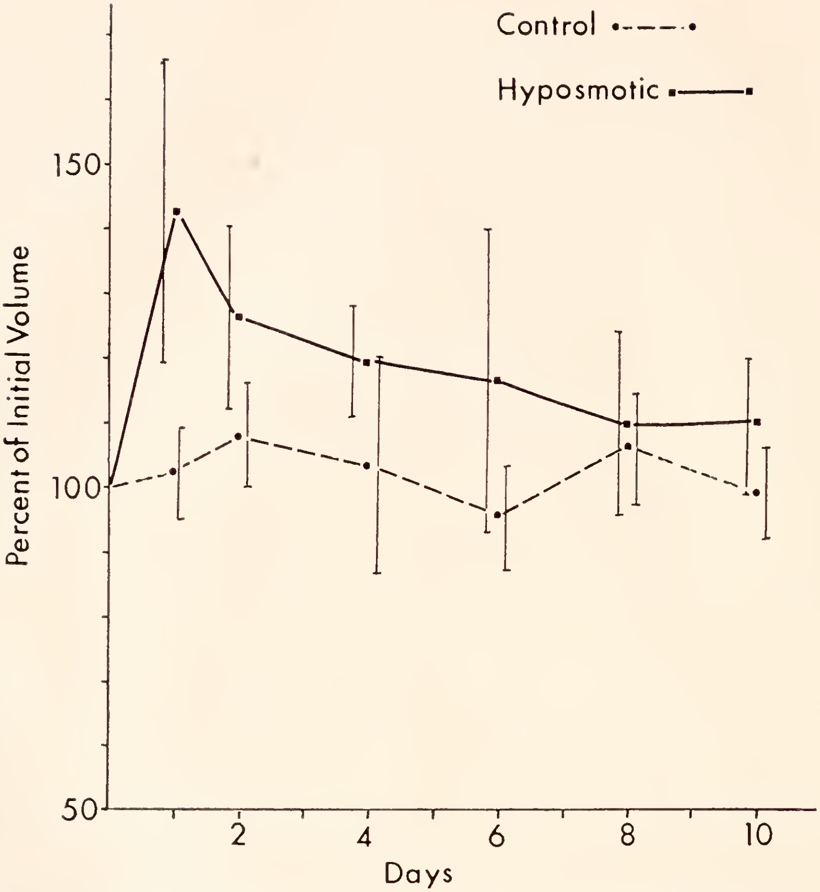

FIGURE 1. Changes with time of the per cents of initial whole animal volume of *Luidia clathrata* maintained at control (ambient) salinity (27‰) and at hyposmotic salinity (16‰). Points represent means (n = 5); vertical lines represent ± 1 s.d.

were removed from the bowls and frozen immediately. The animals were returned to their aquarium until the next incubation period. This procedure avoided the buildup of organic material in the water surrounding the animals which might have occurred otherwise. Ammonia was determined by the microdiffusion method of Conway (1962). Ammonium sulfate was used as the standard. The rate of ammonia excretion was calculated as μg NH_3-N/g dry weight of the animal/hour.

RESULTS

Whole animal analyses

Introduction of *Luidia* into the hyposmotic medium resulted in immediate cessation of movement. Podia were insensitive to mechanical stimuli. Activity of the animals resumed within 48 hours. No deaths occurred in either the hyposmotically stressed animals or the controls in the experiment. The volumes of hyposmotically stressed animals at day 1 were greater than initially (Fig. 1). Subsequently, the per cents of initial volumes declined with time. The per cents of initial volumes of the animals at ambient salinity were close to 100% throughout the experimental period, indicating no significant volume changes. The per cents of the initial volume values of the two groups were significantly different at the 95% level at day 1 but not at subsequent times.

The hyposmotically stressed animals did not right themselves within 10 minutes at the end of day 1 (Fig. 2). The activity coefficients of the hyposmotically stressed animals increased subsequently and became similar to initial levels, indicating recovery of functional well-being.

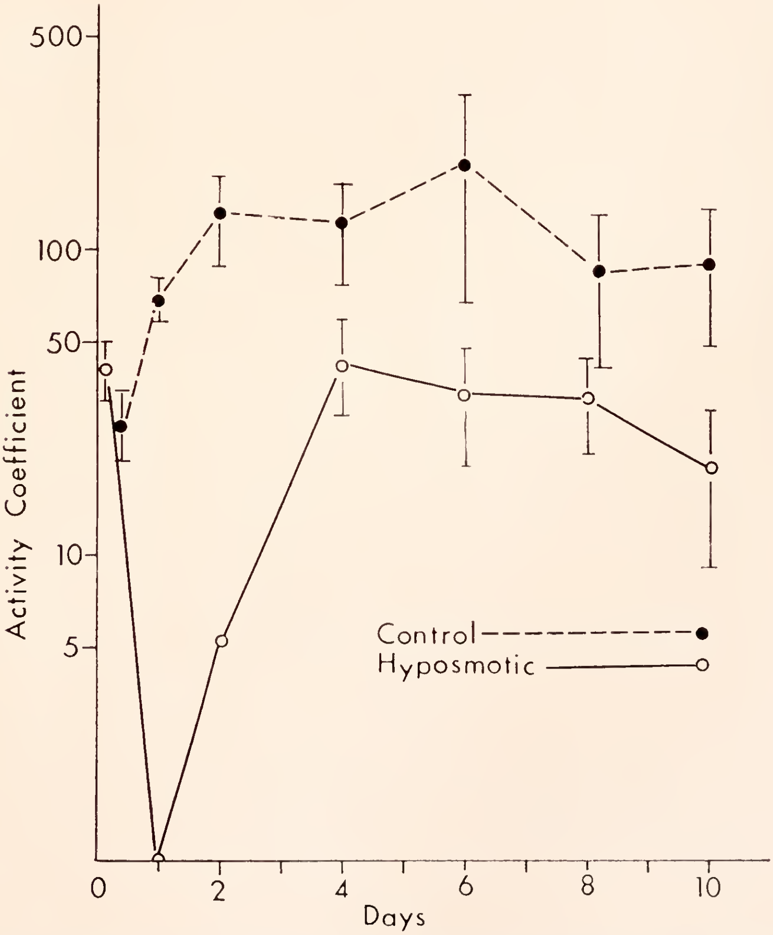

FIGURE 2. Changes with time of the activity coefficients of *Luidia clathrata* maintained at control (ambient) salinity (27‰) and at hyposmotic salinity (16‰). Points represent means (n = 5) except for hyposmotically stressed animals at day 2; vertical lines represent ± 1 s.d. The exception is a median value as one individual failed to right.

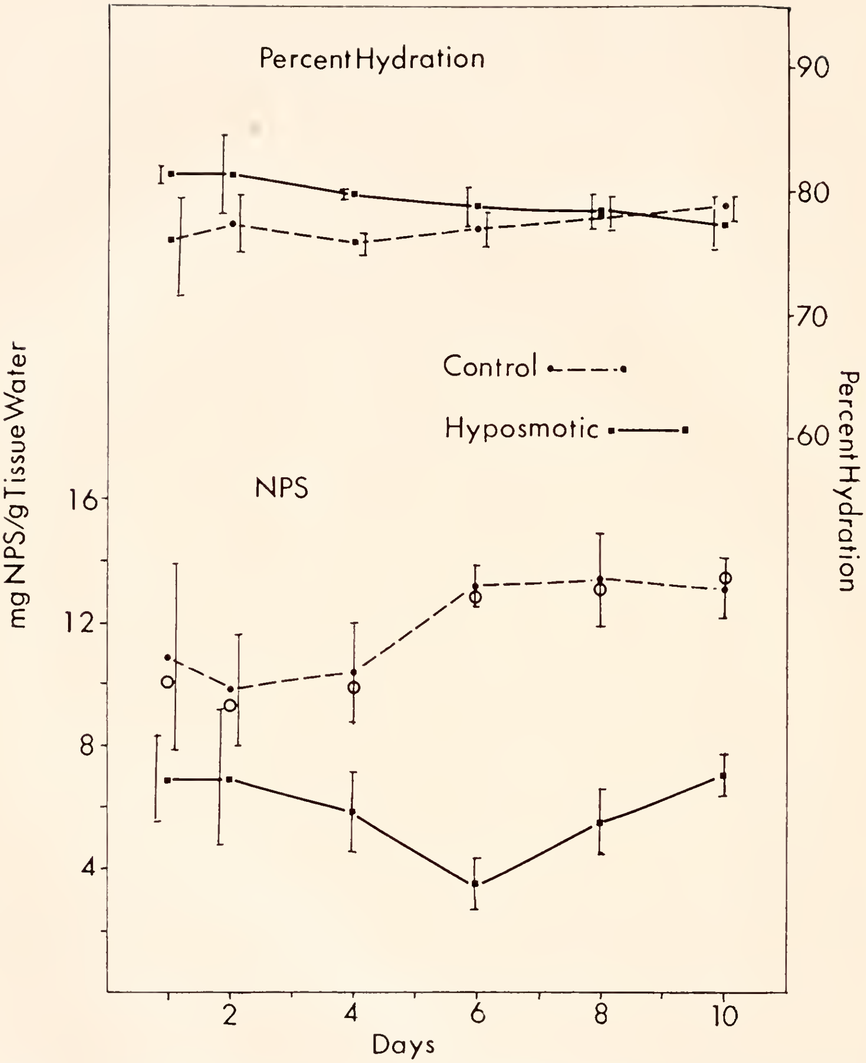

FIGURE 3. Changes with time in the per cents hydration and levels of ninhydrin positive substances (NPS) in the podial tissues of *Luidia clathrata* maintained at control (ambient) (27‰) and at hyposmotic salinity (16‰). Solid symbols represent means (n = 5); vertical lines represent ± 1 s.d. Open symbols represent expected levels of NPS calculated on the basis of changes in tissue hydration.

Tissue analyses

At the end of day 1, the per cents hydration of the podia and the pyloric caeca were significantly higher at the 95% level than those in the tissues of control animals. The per cent hydration of the podia of hyposmotically stressed animals decreased after the first day, eventually equalling the levels in the tissues of control animals (Fig. 3). The per cent hydration of the pyloric caeca of hyposmotically stressed animals decreased after the first day, but remained higher than the levels in the tissues of the control animals (Fig. 4).

The changes in the per cent hydration of both tissues were paralleled by changes in the levels of NPS. The levels of NPS in the podia and the pyloric caeca of hyposmotically stressed animals were lower than those in the tissues of control

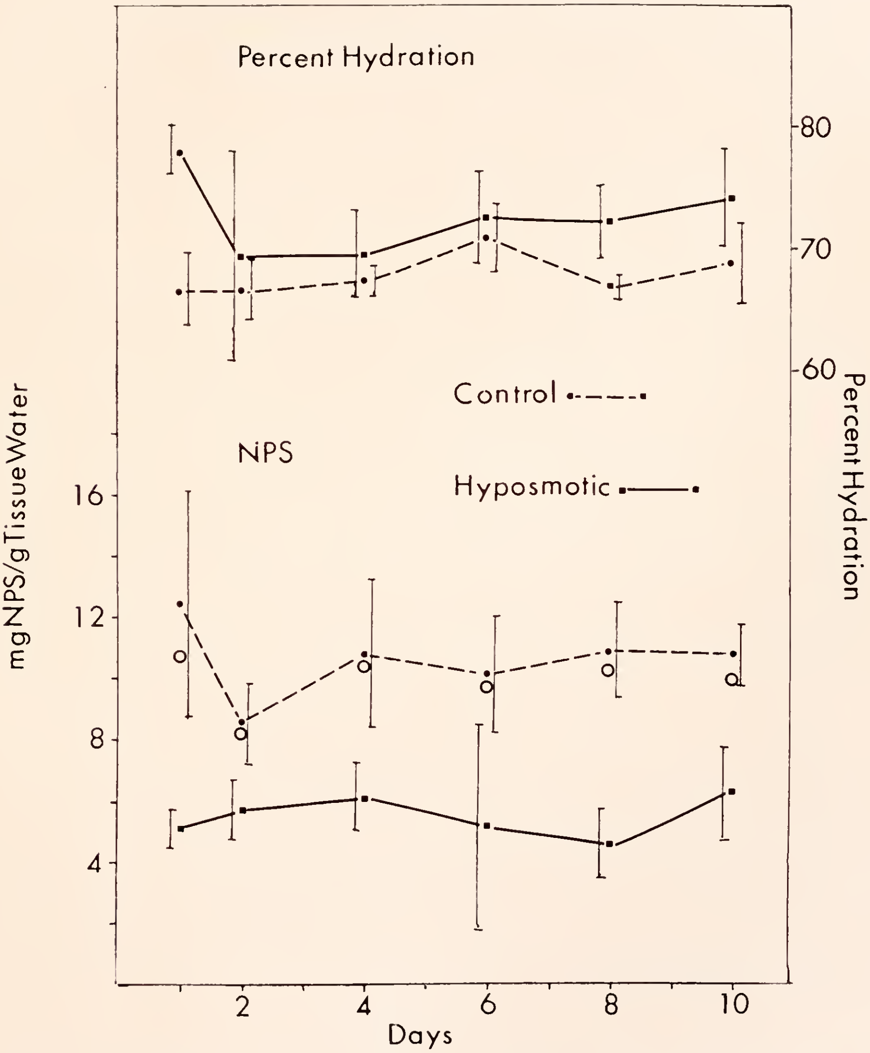

FIGURE 4. Changes with time in the per cents hydration and levels of ninhydrin positive substances (NPS) in the pyloric caeca of *Luidia clathrata* maintained at control (ambient) salinity (27‰) and at hyposmotic salinity (16‰). Solid symbols represent means (n = 5); vertical lines represent ± 1 s.d. Open symbols represent expected levels of NPS calculated on the basis of changes in tissue hydration.

animals by the end of day 1, and remained lower throughout the experimental period. Except for the level in the podia on day 2, the differences in the levels of NPS in both tissues of the stressed animals were significantly different at the 95% level from the levels in the tissues of control animals. Expected changes in NPS levels calculated on the basis of changes in tissue hydration constituted only a small fraction of the decrease actually found in the hyposmotically stressed specimens of *Luidia* (Figures 3 and 4, Table I). Protein levels in the podia and pyloric caeca were 373 mg/g dry weight and 285 mg/g dry weight respectively at the beginning of the experiment. There were little changes in these levels in tissues of either the control or hyposmotically stressed animals.

Ammonia excretion

Ammonia excretion by hyposmotically stressed animals was higher than by control animals throughout the 52 hours of measurement (Fig. 5). The peak of ammonia excretion occurred 26 hours after the initiation of stress. At this time, the mean value of ammonia excretion by hyposmotically stressed animals was almost double that by control animals. The excretion rates declined after this time in the hyposmotically stressed animals and approached the rate of the control animals at 52 hours. There was considerable variation in excretion rates of control and hyposmotically stressed animals. As a consequence, the mean excretion rates of the two groups were not significantly different at the 95% level except for the 26 hour sampling time.

TABLE I

Changes with time in the expected and observed levels of ninhydrin positive substances (mg NPS/g tissue water) in the podia and pyloric caeca of hyposmotically stressed Luidia clathrata. *Expected values were calculated on the basis of the NPS levels in control tissues and on the increase in hydration in hyposmotic tissues. Observed values are means ± s.d. (n = 5)*

Sampling time	1 Day	2 Days	4 Days	6 Days	8 Days	10Days
Podia						
Expected NPS	10.18	9.38	9.89	12.92	13.28	13.34
Observed NPS	6.82	6.96	5.89	3.53	5.57	6.92
s.d.	1.40	2.17	1.35	0.79	1.07	0.75
Pyloric caeca						
Expected NPS	10.66	8.21	10.43	9.87	10.10	9.71
Observed NPS	5.07	7.52	6.12	5.13	4.46	6.22
s.d.	0.58	0.95	1.15	3.38	1.16	1.51

DISCUSSION

An increase in the whole animal volume of hyposmotically stressed specimens of *Luidia clathrata* can be related in part to an increase in tissue hydration. The extent of the increase, however, indicates that most of the volume increase results from the dilution of the coelomic fluid. The subsequent decline in volume of hyposmotically stressed animals with time suggests a degree of volume regulation similar to that found by Virkar (1966) for the sipunculid *Golfingia* and by Pierce (1971b) for two species of the bivalve *Modiolus*. This observation of volume regulation by specimens of *Luidia* contrasts to its reported lack in the echinoid *Strongylocentrotus purpuratus* (Giese and Farmanfarmaian, 1963) and in *Asterias rubens* from a relatively stenohaline population (Binyon, 1961). Binyon (1972a) suggests that earlier reports that *A. rubens* showed volume regulation to be due to mechanical damage induced in handling. There was no observation of such damage to the integument of specimens of *Luidia* in this experiment. The asteroid, *Odontaster validus*, has the ability to retain its original weight in 50% sea water (Pearse, 1967). Thus, there may be at least a limited capacity for regulation of the coelomic fluid in some asteroids. Binyon (1972a) suggests that there may be an optimal coelomic volume to body volume ratio which tends to be maintained.

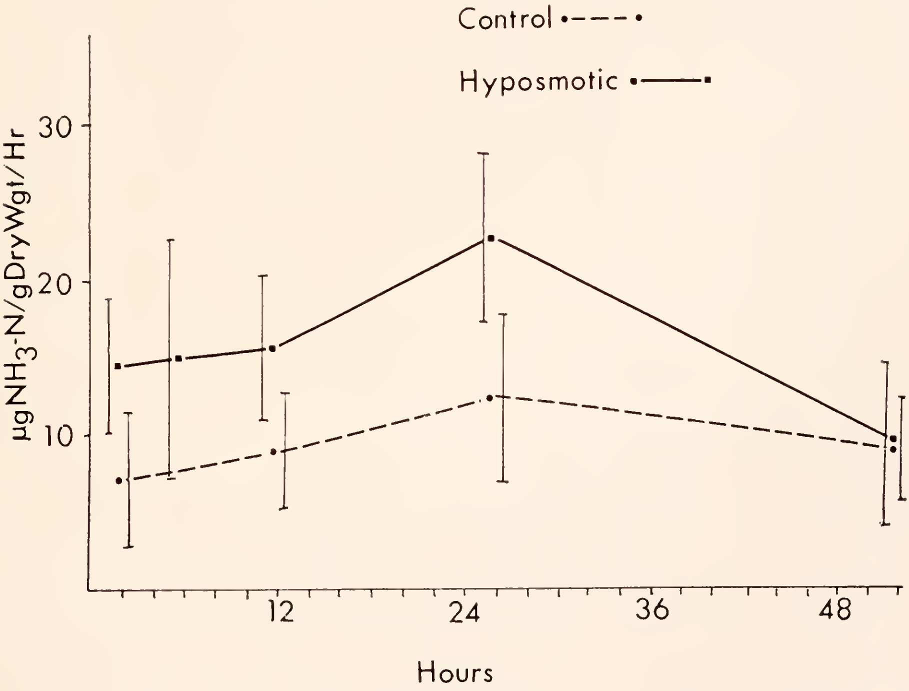

FIGURE 5. Changes with time in the rates of ammonia excretion of *Luidia clathrata* maintained at control (ambient) salinity (27‰) and at hyposmotic salinity (16‰). Points represent means (n = 5); vertical lines represent ± 1 s.d.

As indicated by the effect on the activity coefficient, hyposmotic stress on specimens of *Luidia* results in a temporary loss of locomotory capacity. The ability to recover quickly locomotor activity after dilution of its medium obviously has important ecological significance for this species in bays where such effects can occur suddenly. The ability to recover locomotor activity with hyposmotic stress has been reported for *A. rubens* from the coast of France (Jeuniaux *et al.*, 1962).

Binyon (1972b) reported that a population of *A. rubens* from the coast of Great Britain had a much less capacity to recover locomotor ability. Recovery of activity in hyposomotic conditions has also been found for the echinoid, *S. droebachiensis* (Lange, 1964).

The increase in tissue hydration levels found here for specimens of *Luidia* is similar to that reported for other echinoderms. Kowalski (1955) found that the per cent hydration of ovarian tissue of *A. rubens* was lower in animals from the North Sea (30–32‰) than from the Baltic Sea (15–17‰) with mean per cent hydration values of 80.5 and 84.0 respectively. It has been shown experimentally that the per cent hydration increases with hyposmotic stress in the pyloric caeca (Jeuniaux *et al.*, 1962) and podia (Binyon, 1972b) of *A. rubens*. In both cases, it was concluded that the smaller than expected increase in tissue hydration was related to isosmotic intracellular regulation. The per cent hydration of the gut of the echinoid, *S. droebachiensis*, is increased with hyposmotic stress (Lange, 1964). Lange (1970) stated that water molecules must replace free amino acids lost during cell volume regulation. Thus, the per cent hydration levels of tissues of hyposmotically stressed animals should be somewhat higher than levels of control animals if there is complete adjustment. The amount of decrease in the level of hydration of podia of hyposmotically stressed specimens of *Luidia* is not in accord with this generalization. Variability resulting from the method of absorbing the ambulacral fluid may be responsible for the discrepancy.

The decrease in levels of NPS within 24 hours after the beginning of hyposmotic stress indicates a removal of these molecules from the cells of this species. Virkar and Webb (1970) also found that the absolute response of the free amino acid pool of the hyposmotically stressed clam, *Mya*, is immediate. As the decrease in NPS is far greater than that predicted on the basis of changes in per cent hydration of the tissues of *Luidia clathrata*, the decrease in NPS represents an actual loss and not a dilution of the free amino acid pool. This decrease in the pool has been shown for several bivalve species (Virkar and Webb, 1970; Pierce, 1971a).

A decrease in the levels of tissue NPS with hyposmotic stress has also been reported for the pyloric caeca (Jeuniaux *et al.*, 1962) and the podia (Binyon, 1972b) of *A. rubens* and the gut of the echinoid, *S. droebachiensis* (Lange, 1964). These workers concluded that amino acids were the prime effectors of the isosmotic intracellular regulation in these echinoderms. The change in the NPS levels in the pyloric caeca of specimens of *Luidia* were in proportion to the dilution of the medium, but the changes in the podia were greater than would be expected for compensation. Lange (1964) found that the intestinal levels of NPS in *S. droebachiensis* were a linear function of sea water concentration. Virkar (1966) found a non-linear response of NPS to sea water concentration in the sipunculid, *Golfingia*. Analysis of the levels of NPS in the podia of specimens of *Luidia* at more salinity levels is necessary to ascertain if a non-linear response is occurring here also.

A possible route for the loss of free amino acids in this species would be the deamination and catabolism of the carbon skeletons. The increase in the rate of ammonia excretion in hyposmotically stressed specimens of *Luidia* suggests that this may be occurring. An increase in ammonia excretion associated with hyposmotic stress has been reported in the holothurian, *Eupentacta quinquesemita* and the echinoid, *S. droebachiensis* (Emerson, 1969). The increase in ammonia excretion in *Luidia clathrata* is immediate, as has been found also in the polychaete, *Nereis virens*, and the shore crab, *Carcinus maenas* (Haas, Haberfield and Hammen, 1972).

Schoffeniels and Gilles (1972) suggest that the level of organic effectors is regulated by a control of the synthesis-degradation equilibrium. An activation of enzymes involved in degradation would result in an increase in ammonia excretion. It is possible that hyposmotic stress may result in the activation of specific enzymes involved in amino acid catabolism. An appropriate class of enzymes are the L-amino acid oxidases. Activation of these enzymes of oxidative deamination would result in a decrease in the free amino acid pool, an increase in ammonia excretion, and possibly the often observed phenomenon of an increase in oxygen consumption. Preliminary measurements indicate that there are negligible amino acid deaminases in the podia and pyloric caeca of specimens of *Luidia* (F. E. Friedl, unpublished data). This does not exclude the possibility of highly specific deaminating enzymes. Simpson, Allen and Awapara (1959) reported high concentrations of glycine and taurine in this species. Osmotic stress may result in the activation of enzymes involved in the relatively complex degradative pathways of these two compounds. Since an increase in ammonia excretion with hyposmotic stress occurs in a number of phyla, there may be a uniformity in the molecular events involved in this process.

We conclude that *Luidia clathrata* has two mechanisms which allow it to be euryhaline and able to function in an environment of fluctuating low salinities. These are the ability to partially regulate the volume of its coelomic fluid and to carry on intracellular isosmotic regulation. *Luidia clathrata* apparently is the only member of its genus to be euryhaline. Another member, *L. sarsi,* is stenohaline. Ursin (1960) reported that it is never found at salinities below 30‰. Lower salinities may diminish or even exterminate populations of *L. sarsi* (Fenchel, 1965). The occurrence of euryhalinity in *L. clathrata* is of significance in that it indicates that even an evolutionary primitive group of the class Asterozoa has the capacity to develop this ability.

We thank Dr. F. E. Friedl of the University of South Florida for performing the enzyme assays and for stimulating ideas concerning the molecular basis of the phenomena. We also thank K. Ellington and E. Johnston for their technical assistance.

SUMMARY

(1) The asteroid *Luidia clathrata* becomes inactive when transferred from 27‰ sea water to 16‰, but recovers its activity within 48 hours.

(2) The volume of intact animals increases with the initiation of the hyposmotic stress, but returns to somewhat above the original level within several days. This suggests regulation of the volume of the coelomic fluid.

(3) The changes in the per cent hydration and level of ninhydrin positive substances in the podia and pyloric caeca of hyposmotically stressed animals indicate that intracellular isosmotic regulation occurs.

(4) The increase in the rate of ammonia excretion of hyposmotically stressed animals suggests that the decrease in tissue NPS levels results from their catabolism.

(5) *Luidia clathrata* is a euryhaline echinoderm, responding to reduced salinities by coelomic fluid volume regulation and isosmotic intracellular regulation.

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SHELL SELECTION AND AGGRESSIVE BEHAVIOR IN TWO SYMPATRIC SPECIES OF HERMIT CRABS

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There is abundant evidence that hermit crabs do not enter gastropod shells at random but select shells according to species type and associated characteristics of shape, shell covering, dimension and weight. Reese (1962a, 1963) and Orians and King (1964) concluded from investigation of shell selection behavior in several species of hermit crabs that both preference for and availability of different mollusc shells influence the frequency distribution of shell types occupied. Shell configuration, aperture size, shell weight/crab weight indices and shell weight/shell volume indices, *etc.* (Markham, 1968; Hazlett, 1970b) all provide important evaluation stimuli for selection. Shell entering behavior is elicited through tactile and proprioceptive stimuli from mechanoreceptors on chelipeds and periopods which trigger a sequential series of fixed motor patterns. Reese (1963) and Hazlett (1971) present evidence that such patterns develop in glaucothoe larvae at the time of their initial entrance into shells. Kinosita and Okajima (1968) have described a series of behaviors in the land hermit crab, *Coenobita rugosus,* in which "measurements" of shell aperture by probing and extension of chelipeds provide crabs with information for selection of larger shells as they grow.

In aggressive encounters which may be associated with competition for suitable shells and which may result in establishing dominant-subordinate relationships, hermit crabs display characteristic patterns of behavior. These have been analyzed in detail by Hazlett (1966a, 1967, 1968a, 1968b, 1970c, 1972a) and by Hazlett and Bossert (1965, 1966). Elevation of the body and extension of chelipeds provide effective signals for altering behavior in combatants. Increase in weight and visual shell size enhances the probability of a crab initiating or dominating an encounter although when size differences are pronounced larger animals tend to ignore display by smaller individuals. Shell size has no effect on the level of aggressiveness in *Pagurus hirsutiusculus* according to Vance (1973b) although Hazlett (1970b, 1970c) found that occupancy of inappropriately sized shells increased aggression in both *P. bernhardus* and *Clibanarius vittatus* individuals. Aggressive contact may be inter- or intra-specific depending on the species involved. According to Reese (1962b, 1964) attacks may be prevented if subordinate crabs assume submissive postures. The probability of a crab winning an encounter is influenced by its past history of success or failure as shown by Hazlett (1966b) and Mainardi and Rossi, (1971). According to Rossi (1971) dominant crabs are larger, obtain more food and molt more frequently. The investigations of Courchesne and Barlow (1971) and Hazlett (1966b, 1968c) have shown that isolation increases and aggregation decreases aggression, although agonistic behavior may increase with initial crowding until crabs have adjusted to new density levels.

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In the sub-littoral zone of Frenchman Bay along the northeast shore of Mt. Desert Island, Maine two species of hermit crab *Pagurus acadianus* and *Pagurus pubescens* occur sympatrically. A ten-year survey (Grant, 1963 and more recent unpublished data) has shown that since 1967 *P. pubescens* has infiltrated areas just below low-tide level previously occupied exclusively by *P. acadianus*. The present studies were undertaken to investigate what role if any shell selection, shell availability and agonistic behavior may play in furthering coexistence or competitive exclusion (Gause, 1934) at the interspecific level.

Materials and Methods

Crabs were obtained by diving at three sites: Salisbury Cove and Laboratory Cove on the northeast shore of Mt. Desert Island and at Sullivan Harbor on the north shore of Frenchman Bay. Shells occupied by crabs were sorted into four, arbitrary size categories and the number in each set and the types of shells occupied recorded. Shell aperture diameter, measured at right angles to the columellar axis, was used at the basis for defining each set: small (1.0–9.9 mm), small intermediate (10.0–14.9 mm), large intermediate (15.0–19.9 mm) and large (above 20 mm). Correlation coefficients for regression curves calculated for crab weight against aperture diameter were highly significant (0.743, $P < 0.001$ and 0.636, $P < 0.001$ for *P. acadianus* and *P. pubescens* data respectively) so that division of crabs into any series of sets on the curve resulted in relatively slight overlap values between adjacent sets. Although other dimensional correlations may be more significant (Hazlett, 1970b) the one adopted proved a valid and convenient method for estimating crab size without the necessity of evicting animals from their shells subsequent to initial determinations. The shell adequacy index of Vance (1973a, 1973b) would have provided a more rigorous standard. One hopes that this index will be generally adopted for hermit crab studies, particularly in cases where crabs occupy a wide range of domicile shell types.

In field studies fifteen, square foot, bottom samples were taken along roughly determined "swim" lines at each collecting site in order to estimate the supply of empty mollusc shells. More complete analysis was hampered by strong tidal currents and low water temperature (11° C).

Experimental animals were held in circulating sea water tanks at 11° C–13° C and fed regularly with shredded mussel and clam. For some experiments ten to twelve crabs were crowded into 15 × 21 sq. cm plastic containers from 60 to 108 hours prior to testing or individually isolated in the compartments of plastic, perforated ice-cube trays covered with sheets of perforated plexi-glass in running sea-water. All behavioral tests were conducted in 35 × 35 sq. cm polythene trays provided with running sea-water and a substratum of washed gravel.

For purposes of uniformity, most tests were run with crabs in the small intermediate set which contained animals with a shell aperture range between 10.0 and 14.9 mm and average crab weight/shell aperture indices of 3.16 ± 0.83 for *P. acadianus* and 2.68 ± 0.61 for *P. pubescens* collections. Crabs were generally selected from the 11–14 mm aperture range in order to avoid as many overlaps with other sets as possible. As preliminary tests showed none of the heightened aggressiveness of small females reported by Reese (1962a) for other species of hermit crabs, animals were not sexed in the following investigations. No subject was used more than once in any of the trials reported below. Where necessary crabs were evicted from their domicile shells by applying an electric soldering gun at the shell apex.

Experiment I

In order to test the influence of shell abundance on shell selection, small intermediate sized crabs were evicted from domicile shells and allowed to enter *Littorina* shells whose aperture measurements were appropriate to crabs of the smallest size range. *Littorina* and *Thais* shells were arranged at equal intervals around a perimeter surrounding a centrally placed container from which crabs were released into the test chambers. The ratio of *Littorina* to *Thais* shells varied in the different tests, as follows: 5:2, 2:5, and 4:4. Each test was terminated at twelve hours and the type of shell occupied by the crab recorded. Previous experience indicated that crabs seldom exchanged shells after a twelve-hour period under similar conditions.

Experiment II

Tests were conducted to see if a difference existed between the two crab species in their preference for shells colonized by the hydroid *Hydractinia echinata*. In one group of trials crabs evicted from *Littorina* domicile shells were given the choice of entering either of two *Littorina* shells of the same size, one bare and one covered with a *Hydractinia* colony. In each of second series of trials a crab with a bare domicile shell was released in the presence of an empty, *Hydractinia*-colonized shell. All of the above trials terminated after twelve hours when the type of shell occupied was recorded.

Experiment III

Investigations were designed to compare conspecific aggression in the two crab species. In each trial a pair of crabs was released in the test area for a ten-minute period, this being sufficient time for crabs to establish dominant/subordinate relationships. Dominant crabs showed aggressive display by extending their chelipeds forward, by vertical rotation of their shells and by rising on their ambulatory legs. Dominance was considered to be established when one crab of a pair persistently retreated at the advance of the other or was repeatedly forced into submissive positions. Threat and intimidation often were followed by direct assault which resulted in the eviction of subordinate individuals from their shells in a manner similar to the behavior reported by Hazlett (1967, 1970a, 1972b) for a number of hermit crab species including *Pagurus pubescens*. In the present tests eviction positioning was recorded as additional evidence of dominance and trials were concluded before the end of the test period as soon as this position was assumed by the dominant crab.

In another series of tests crabs which had been either isolated or crowded from 60 to 108 hours were tested for dominance in trials each of which utilized one isolate and one recently crowded individual. In another group, dominance

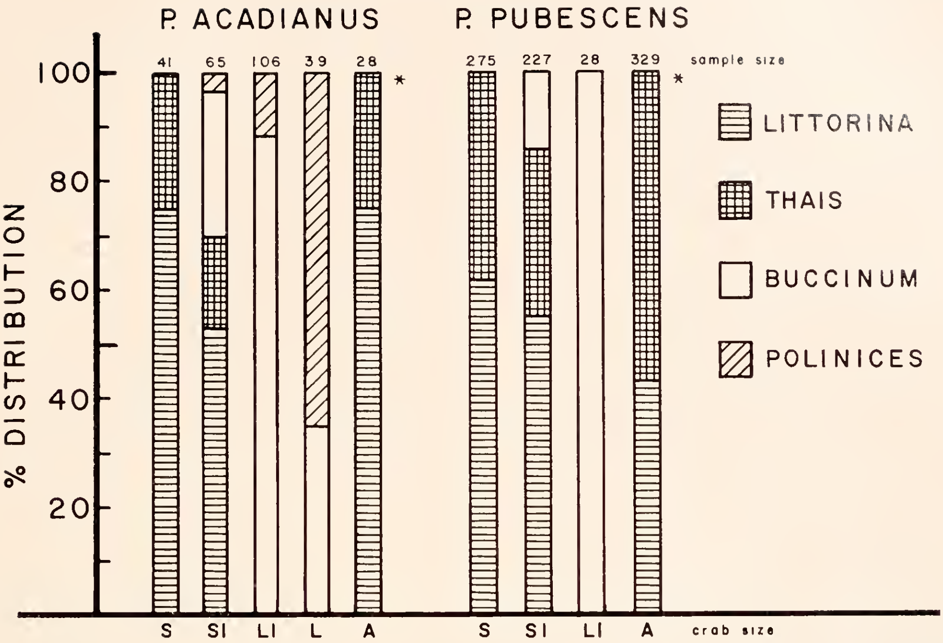

FIGURE 1. Per cent distribution of mollusc shell types occupied by different size categories of *Pagurus acadianus* and *P. pubescens* from pooled samples and collections from Sullivan Harbor(*). Crabs from the latter area were with few exceptions in the small and small-intermediate size range. Abbreviations for size categories are: S, small; SI, small intermediate; LI, large intermediate; L, large; A, all sizes.

tests were performed between animals occupying different shell sizes relative to their body weights. Dominance was recorded between crabs in normal size shells (appropriate crab weight/shell volume or aperture index) and individuals of the same size range housed in shells of inadequate dimensions *or* between animals occupying small and large shells (high and low crab weight/shell volume index respectively).

RESULTS

Field data and observations

The distribution of shell types occupied by different size groups of *P. acadianus* and *P. pubescens* from pooled samples of all collecting sites and from the Sullivan Harbor collections is shown in Figure 1. Shells principally occupied by crabs were *Littorina littorea*, *Thais lapillus*, *Buccinum undatum* and *Polinices heros*. In both species individuals in the two smallest size groups were found principally in *Littorina* and *Thais* shells. The overlap value in terms of the proportion of shells occupied in common by crabs of each species was 84%. Larger crabs occupied shells of *Buccinum* and *Polinices* at a lower overlap value of 42%. These data support previous analyses by Lindsey and Grant (1970). It is important to note the relative numbers of shell types within each category of crab size, and to compare data from pooled samples with those from Sullivan Harbor. At this

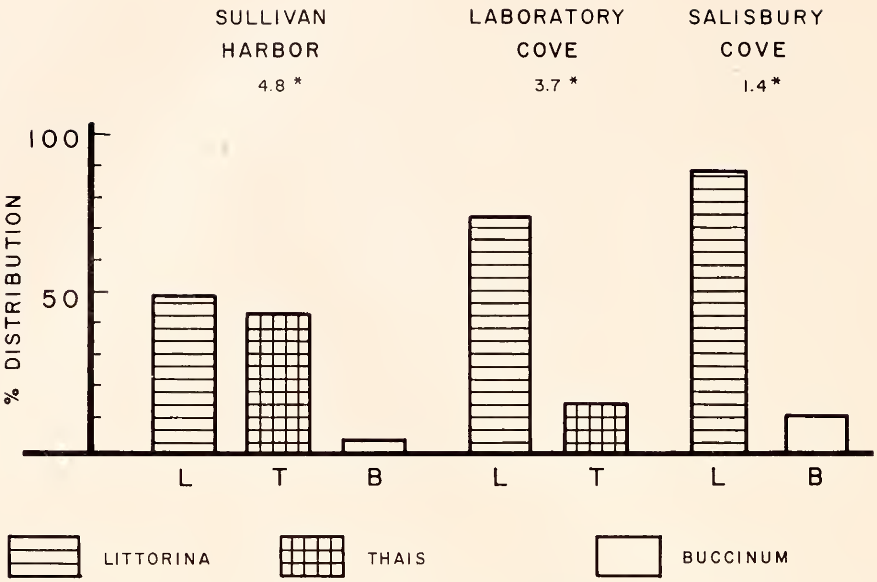

FIGURE 2. Average per cent distribution of empty mollusc shells collected in fifteen square foot samples from each of three localities in Frenchman Bay. Ninety-six per cent of all shells collected were in the smallest size range. Asterisk indicates mean number of empty shells per square foot.

location a higher proportion of the *P. pubescens* population occupied *Thais* shells than elsewhere, relatively few specimens of *P. acadianus* were collected and an overlap value of 63% was recorded. Twenty-seven per cent of the *P. acadianus* specimens were found in shells covered with colonies of the hydrozoan, *Hydractinia echinata*. Only one individual of *P. pubescens* was recorded in a *Hydractinia* covered shell out of 529 captures. It is interesting to note that hydrozoans were not found on any shells containing living molluscs within the area of study.

Field observations at a number of locations indicated that individual, larger sized *P. acadianus* are widely spaced. They tend to occupy elevated areas such as rocks which protrude from a surrounding area of low sand or low gravel. *P. pubescens* and small *P. acadianus* individuals on the other hand, most commonly were found closely spaced in areas around wharf pilings and on vertical faces of rock wall though *P. acadianus* individuals appeared more widely dispersed. In such assemblages crabs do not appear to maintain individual distances as such or to form closely spaced aggregates.

Most empty shells collected in the field survey were small size *Littorina* and *Thais* shells (Fig. 2). Their density was highest at Sullivan Harbor where the proportion of *Thais* to *Littorina* shells was greater than in Laboratory Cove. No *Thais* shells were collected in Salisbury Cove.

Behavior studies

Experiment I. The results of tests conducted to see if relative abundance of different types of shells influences crab selection are given in Table I. *P. acadianus*

TABLE I

Shell availability studies: results of twelve hour selections by individual crabs presented with empty mollusc shells in different species ratios. All animals were small intermediates: P values determined by χ^2

Pagurus acadianus		Shell selection	
Shell ratios *Littorina/Thais*	No. trials	*Littorina*	*Thais*
5/2	12	11	1
2/5	12	9	3 $P < 0.05$
4/4	13	11	2 $P < 0.02$
Pagurus pubescens			
5/2	10	7	3
2/5	12	2	10
4/4	15	9	8

test animals chose *Littorina* shells more often than could be predicted on the basis of shell-type ratios except in those trials where *Littorina* shells were the most abundant type, whereas *P. pubescens* individuals chose shell types in proportion to their relative abundance.

Experiment II. Table II gives the results of experiments which tested the preference of individual crabs evicted from their domicile shells when given the choice between a bare shell and one *Hydractinia* colonized shell. The number of trials in which crabs chose shells with *Hydractinia* colonies was significantly higher for *P. acadianus* and significantly lower for *P. pubescens* test animals than could be predicted on the basis of random selection.

In each of the second trial series, individuals of *P. acadianus* were recorded in colonized shells at the end of the twelve hour test period in all but two cases, while only one *P. pubescens* crab abandoned its domicile shell (Table III). Considering the preference shown by evicted crabs for previously occupied domicile shells (Grant, 1963), the large number of *P. acadianus* individuals which abandoned domiciles in the above trials supports the view that the species has a strong preference for shells colonized by *Hydractinia.* The species of shell colonized did not appear to affect the results.

Experiment III. As pilot tests demonstrated little agonistic behavior at the inter-specific level between individuals of *P. acadianus* and *P. pubescens,* the experi-

TABLE II

Selection by evicted crabs of shells with and without Hydractinia *colonies.*

	No. trials	Select colonized *Littorina* shell	Select bare *Littorina* shell	Sign test
P. acadianus	39	29	10	$P < 0.02$
P. pubescens	40	10	30	$P < 0.02$

TABLE III

Selection of shells with Hydractinia *colonies. Each trial represents a choice made by a crab in its home shell presented with a* Hydractinia *covered shell of appropriate size:*
L = Littorina; *T* = Thais; *B* = Buccinum.

No. trials	Home shell type	Colonized shell type	No. selecting home shell	No. selecting colonized shell
		P. acadianus		
15	B	B	2	13
10	L	L	0	10
3	L	P	0	3
		P. pubescens		
6	B	B	6	0
12	L	L	11	1
4	T	L	4	0
4	L	T	4	0

ments reported below were limited to comparisons of conspecific aggression in the two species.

Isolated individuals of *P. acadianus* showed a significantly higher incidence of dominance over crowded individuals in most trials and assumed eviction positions in nearly half of those trials in which they were dominant (Table IV). That the period of isolation may effect level of aggressiveness is suggested by the fact that the percentage of 108 hour isolates achieving dominance and taking eviction positions was slightly higher than for those isolated 60 hours, although the differences were not significant. No agonistic behavior was recorded in any of the trials

TABLE IV

Results of conspecific dominance tests between isolated and crowded individuals

	No. trials	No. isolates dominant	No. crowded dominant	P (sign test)	No. isolates assuming eviction position
			Pagurus acadianus		
Pooled data	44	32 (72.72%)	11 (25.00%)	$P < 0.01$	13 (43.75%)
60 hr	25	17 (68.00%)	8 (32.00%)	$P < 0.05$	5 (28.82%)
108 hr	19	15 (78.04%)	3 (15.78%)	$P < 0.01$	8 (53.33%)
			Pagurus pubescens		
60 hr and 108 hr	34	No agonistic behavior recorded between isolated and crowded individuals			

Dominance not established in one trial.

with *P. pubescens* crabs, suggesting that the level of aggression, in the species is influenced neither by isolation nor aggregation.

The results of another series of dominance tests in which test animals occupied different shell sizes relative to body weight are shown in Figure 3. Most individuals of both crab species which occupied inadequately small shells (high crab weight/shell volume index) were dominant over animals housed in domicile shells of adequate size. Dominance was evenly distributed in tests between individuals of *P. acadianus* occupying normal and overly large shells. No agonistic behavior at all was recorded for individuals of *P. pubescens* in similar trials. In other tests of this series in which dominance was recorded between paired crabs occupying small and large shells, *P. acadianus* crabs displayed agonistic behavior in every trial and dominance ranking between small and large shelled individuals was approximately even. However, small-shelled crabs of *P. pubescens* were dominant in most trials and half of the dominant individuals assumed eviction positions.

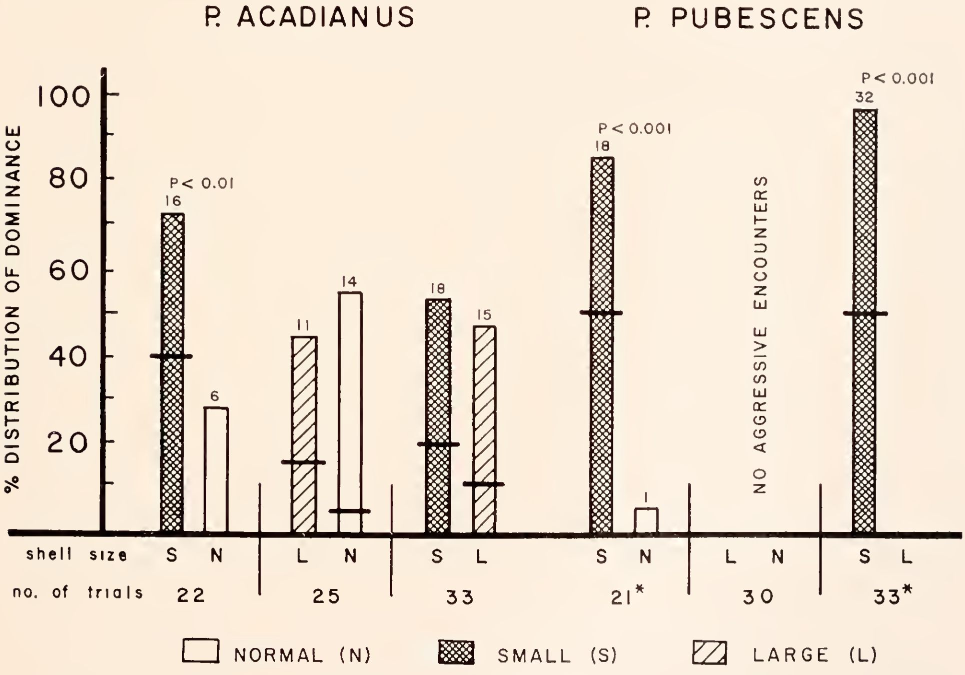

FIGURE 3. Results of con-specific dominance tests in *Pagurus acadianus* and *Pagurus pubescens* between paired individuals in different size shells according to crab weight/shell volume indices. The proportion of trials in which dominant crabs assumed eviction positions is shown by the area below the heavy horizontal lines on the graphs. In some trials no aggressive behavior was recorded.

DISCUSSION

It is important to consider the above results in terms of the relative impact which hermit crab behavior may have on influencing ecological overlap between the two species under study. Such discussion assumes that mollusc shells constitute an essential resource to the crabs which is roughly analogous to territories, nesting sites, *etc.* of other animals.

On the basis of crab weight/shell aperture indices, *etc.,* it is clear that a large proportion of the *P. acadianus* population exceeds the size range of *P. pubescens* individuals in the study areas. The larger specimens of *P. acadianus* principally inhabit *Buccinum* and *Polinices* shells and thus are not in competition with smaller individuals of *P. pubescens* for suitable domiciles. In the smaller size categories *Littorina* and *Thais* are by far the most common genera of shells occupied by both crab species down to the very smallest individuals. Laboratory studies suggest that *P. acadianus* has a distinct preference for *Littorina* shells even if they are in relatively low supply. *P. pubescens,* however, selects shells in rough proportion to their relative abundance suggesting that shells constitute a "fine-grained" resource (Levins, 1968) for this species. Thus where *Littorina* provides a single resource for domicile shells the two crab species may be in direct competition. Where both *Littorina* and *Thais* shells are available both species may coexist but where *Thais* shells predominate *P. pubescens* could have a competitive advantage. Although field surveys were not extensive enough to be conclusive they tend to support this general assumption. In Sullivan Harbor where the density of *Thais* shells was highest the number of *P. pubescens* was proportionately greater and a larger percentage of the population occupied *Thais* shells than elsewhere. This suggests that resource partitioning may be a more important mechanism furthering coexistence of hermit crab species in Frenchman Bay than in the case of the sympatric species of intertidal crabs studied by Vance (1973a). The preference of *P. acadianus* individuals for *Hydractinia* colonized shells could serve to separate resources and thus favor coexistence with *P. pubescens* even in areas as Salisbury Cove where *Littorina* provides the sole resource of domicile shells.

The failure to find empty shells in large size ranges raises another problem which may be closely related to behavior and individual fitness (Childress, 1972) in *P. acadianus. Buccinum* shells which constitute the major domicile resource of larger crabs are relatively fragile and subject to breakage by wave action. Thus empty shells are limited in supply and those shells already occupied by crabs will constitute the bulk of the available resource. As *Hydractinia* grows slowly and does not occur on shells containing living molluscs in the study area, *Buccinum* and *Polinices* shells colonized by the hydrozoan may constitute natural "heirlooms" which are occupied by successive generations of crabs over a period of years.

Schijfsma (1935) was unable to demonstrate a predeliction for *Hydractinia* covered shells in *P. bernhardus,* a species closely related to *P. acadianus.* However, recent investigations by Jensen (1970) and Wright (1973) working with *P. bernhardus* and with *P. longicarpus* and *P. pollicaris* respectively, confirm our observations that some hermit crabs do show strong, preferential selection for *Hydractinia.* Wright's suggestion that *Clibanarius vittatus* is seldom found in *Hydractinia*-covered shells because crabs are exposed during low tides at temperatures exceeding the tolerance range of the hydroids is not applicable in the case of *P. acadianus* in Frenchman Bay where hermit crabs are never found above the sub-littoral zone even at high tide.

Aggressive behavior is pronounced in *P. acadianus.* This indicates that there may be considerable intra-specific competition for shells particularly among larger crabs, a factor which could act to limit or regulate population growth. The fact that individuals of *P. acadianus* appear to be widely spaced in nature together with the increased ability of isolates to dominate encounters, suggests that maintenance of distance between individuals may be a behavioral strategy adapted to limited shell supplies in larger animals. As Grant (1963) and Berman, Francis and Grant (1973) have shown that this species is moderately errant, individual space rather than territoriality *per se* is probably one significant feature of their population dispersal. It might also be advantageous for aggressiveness to diminish under crowded conditions when crabs congregate on occasion at some common food source, a suggestion which is supported by the above investigations. Hazlett (1968c) has proposed that the increase in density of crabs within an area may come about slowly thus allowing time for adjustment and a decrease in levels of aggression. Specimens of *P. bernhardus* from the Clyde tend to clump together as density increases according to Kenneth Mitchell, University of Glasgow (personal communication). *P. pubescens* on the other hand appears to be a more closely spaced species and exhibits little agonistic behavior following isolation or when crowded. There is, however, no evidence that this species forms social groupings similar to those reported by Rossi (1971) for *Diogenes pugilator*.

According to Vance (1973b) aggressive levels were unaffected by shell size in *Pagurus hirsutiusculus* although the probability of larger crabs evicting smaller individuals during encounters increased as the size of their shells decreased. In *P. pubescens*, however, occupancy of inadequately small shells produces a marked increase in aggressiveness over the low levels usually displayed. Heightening of agonistic behavior also occurs in *P. acadianus* under similar conditions although it is less pronounced in this normally more aggressive species. Increased aggressiveness in hermit crabs which occupy shells too small by volume is understandable as a behavioral strategy to accommodate individual growth and is consistent with Hazlett's (1970b) view that shell volume provides a stimulus to aggression in crabs which have outgrown their domicile shells. Childress (1972) has shown that *Clibanarius albidigitus* individuals tend to "trade up" to shells larger than necessary and that this behavior enhances reproductive fitness based upon increase in clutch size. This, of course, would be most difficult where appropriate shells are limited in supply or are already occupied. Indeed Vance (1973a) has described situations in which larger hermit crabs were found occupying shells significantly smaller than preferred size.

The fact that neither *P. acadianus* nor *P. pubescens* individuals showed an increase in agonistic behavior when housed in large shells is interesting as Hazlett (1970b) found that increase in shell weight stimulated aggression in *P. bernhardus*. As species differences in threshold levels are to be expected it seems likely in the present studies that occupied shells, although large by volume, were not heavy enough to provide sufficient stimulus to increased aggression. In females it may be of selective advantage to avoid unnecessary combats if occupation of shells somewhat larger than necessary does promote an increase in reproductive potential as suggested by Childress (1972).

It seems reasonable to conclude on the basis of the present investigation that differential behavior in shell selection is sufficient to allow co-existence of *P. acadianus* and *P. pubescens* populations under most conditions. Where competition exists, however, it is probably not sustained by inter-specific shell fighting *per se* as described by Hazlett (1970c) in hermit crab species from Hawaii. Agonistic behavior in both species studied is primarily a factor of conspecific competition.

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SUMMARY

Two species of hermit crab, *Pagurus acadianus* and *P. pubescens* are distributed sympatrically in offshore waters of Frenchman Bay, Maine. Laboratory and field investigations indicate that smaller sized specimens of *P. acadianus* have a preference for *Littorina* shells over *Thais* shells whereas *P. pubescens* individuals enter shells of either *Thais* or *Littorina* depending on the relative abundance of each type. Larger *P. acadianus* individuals which usually exceed the size range of *P. pubescens* principally occupy shells of *Buccinum* and *Polinices*. *P. acadianus* shows a strong preference for mollusc shells colonized by *Hydractinia echinata*.

Isolation increases the level of aggression in *P. acadianus* individuals, but the low levels of agonistic behavior found in tests with *P. pubescens* crabs appear not to be affected by changes in crab density. In both species, subjects in shells of too small a size by volume showed a high degree of dominance in trials over crabs occupying normal or large shells; a factor which can be considered an adaptation to accommodate individual growth.

It is concluded that behavior related to shell selection in the two species of crabs is sufficiently different to allow co-existence of the two species on the basis of resource partitioning, except in situations where smaller sized *Littorina* shells without *Hydractinia* colonies constitute a single resource base. However, there may be considerable intra-specific competition between larger specimens of *P. acadianus* for limited supplies of *Buccinum* shells and between crabs of all sizes located in appropriate sized shells and those which have outgrown their domicile shells.

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AN ENERGY BUDGET FOR *MENIPPE MERCENARIA* LARVAE FED *ARTEMIA* NAUPLII

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Only a few of the published reports on the energetics of marine crustaceans include data for larval stages or juveniles (Marshall and Orr, 1955; Corner, Cowey, and Marshall, 1967; Lasker, 1966; Reeve, 1969b; Regnault, 1969; Tsikhon-Lukanina and Lukasheva, 1970; Clutter and Theilacker, 1971), and only one (Tsikhon-Lukanina and Lukasheva, 1970) is complete from time of hatching. In addition, there have been no total energy budgets (meaning all energy sources and sinks accounted for) for commercially important shellfish in the available literature. In the present work we have studied the entire larval life (five zoeal stages and one megalopal stage) of the stone crab, *Menippe mercenaria*. We have accounted for the major flows of energy into and out of the organism through the period of larval development.

The energy balance in an organism may be described using the terms proposed by Petrusewicz and Macfadyen (1970). Matter consumed (C) is either digested (D) or egested (F). Digested material may be assimilated (A) or lost to excretion (U). Energy which has been assimilated is either channeled into energy for maintenance of vital life processes or into production (P), which may be subdivided into growth of new body tissue (P_g), exuvia (E), reproductive products (P_r), and secretions. The former is measured by oxygen consumption and/or carbon dioxide production and is termed respiration (R). In the present study, consumption (C), growth of new body tissue (P_g), exuvia (E), and respiration (R) have been measured. Egestion (F) and excretion (U) are considered together as rejecta (FU) and obtained by subtraction. For larvae, growth of reproductive products is not applicable.

MATERIALS AND METHODS

Ovigerous females were obtained from Florida and held in small recirculating seawater systems. Larvae were collected soon after hatching and placed in finger bowls of filtered seawater at 30.0 ± 1.0‰ salinity. Animals used in the feeding experiments were reared individually in plastic compartmented boxes. Three inch finger bowls with ten zoeae per bowl were used to determine survivorship and molting frequency, and eight inch bowls with up to five hundred larvae per bowl were maintained for growth, respiration, and caloric determinations. All cultures were kept at 25.0 ± 0.5° C on a 12:12 light-dark cycle. Rearing techniques were modified from Costlow and Bookhout (1959). Larvae were changed daily to freshly filtered seawater and fed newly-hatched *Artemia salina* nauplii at a density of approximately 5/ml. Food was always in excess. Number of exuvia present and mortality were recorded daily for all except the mass culture bowls.

Energy consumed was measured daily as the difference between the number of *Artemia* nauplii initially offered to each individual and those remaining after twenty-four hours. Since the concentration of *Artemia* has been shown to affect the rate of development in decapod larvae (Reeve, 1969a, 1969b; Mootz, 1973), each larva was provided with 100 *Artemia* nauplii in 20 ml of seawater. This was consistent with the food density present in cultures used to determine growth, molting frequency, caloric content, and respiration.

Replicate samples of larvae were taken every second day from hatching to day 24, counted, dried at room temperature in a desiccator, and weighed on an analytical balance to the nearest 0.01 mg. The values were plotted on semi-logarithmic paper and a least squares linear regression line fitted to the data. Dry weight data were also obtained for animals that had just molted to the first juvenile crab stage and the exuvia for each stage. Standard deviations and confidence limits were calculated for these data.

Caloric content was determined by wet oxidation in the presence of an acid-dichromate mixture (Maciolek, 1962; American Public Health Association *et al.*, 1965) for larvae at two-day intervals, exuvia of each stage, first juvenile crabs, and newly-hatched *Artemia* nauplii. Standard deviations and confidence limits were calculated for the data. Feeding rates and growth expressed as dry weight were converted to energy units using the values obtained.

Metabolic energy expenditure was measured on a daily basis through megalopa in an all-glass differential microrespirometer. The procedures were modified from Grunbaum, Siegel, Schulz, and Kirk (1955). Readings were taken in the middle of the day to avoid any diurnal effects which were not considered in this study. The experimental animal was placed in the respiration chamber with 0.5 ml filtered seawater, and a piece of filter paper containing 10 μl of 10 per cent potassium hydroxide was attached. The two flasks were connected to a 0.3 mm bore capillary tube. The unit was allowed to equilibrate for 15 minutes after which it was sealed

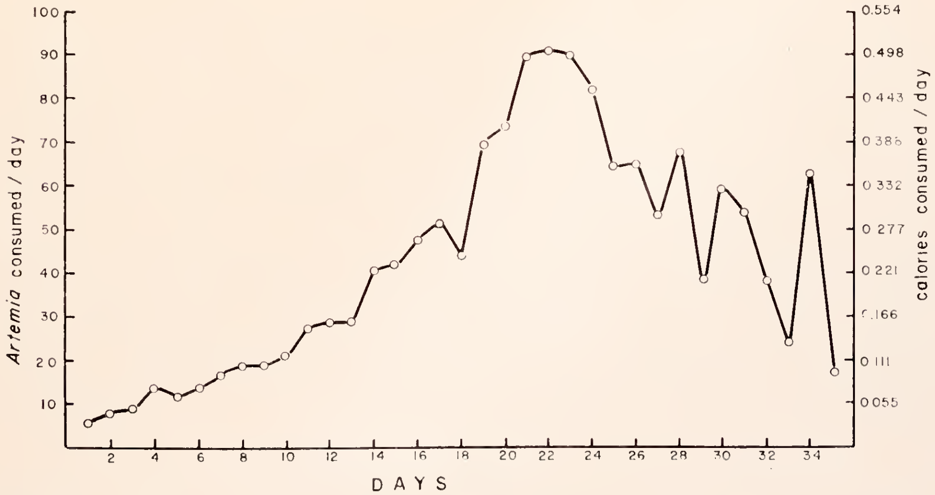

FIGURE 1. Feeding rate for larvae of *Menippe mercenaria* at a density of 5 *Artemia* nauplii/ml.

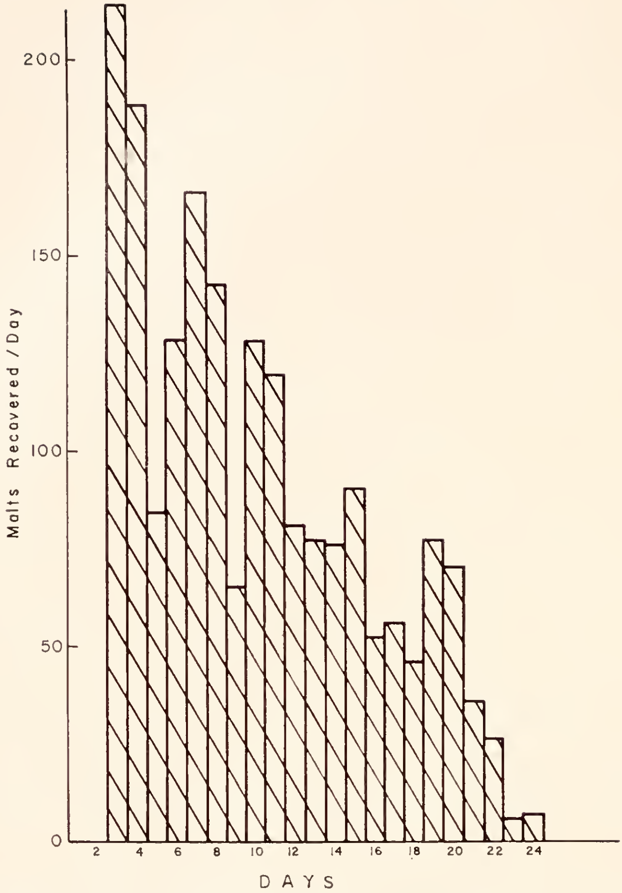

FIGURE 2. Molting frequency of larvae of *Menippe mercenaria* showing duration of each stage.

and placed in a water bath at 25° C. Each daily run consisted of an initial reading and four consecutive hourly readings. The number of larvae used per determination varied as follows: 10 larvae on day 0; 5 larvae on days 2, 3, and 4; 4 larvae on day 5; 2 larvae on day 6; and 1 larva each day thereafter. After four hours, the larvae were removed from the chamber, dried to constant weight in a room temperature desiccator, and weighed on an analytical balance. Animals were not fed during the experiments or for several hours prior to each determination. The results were plotted on double logarithmic paper and a least squares linear regression line fitted to the data for the zoeal stages. Oxygen consumption by the megalopa was averaged and a standard deviation calculated for the data. A caloric equivalent for oxygen consumption of 4.825×10^{-3} calories/μl O_2 consumed was used to convert the data to energy units (Brody, 1945).

RESULTS

The average daily feeding rate is shown in Figure 1. Consumption reaches its peak during megalopa and declines several days prior to the onset of meta-

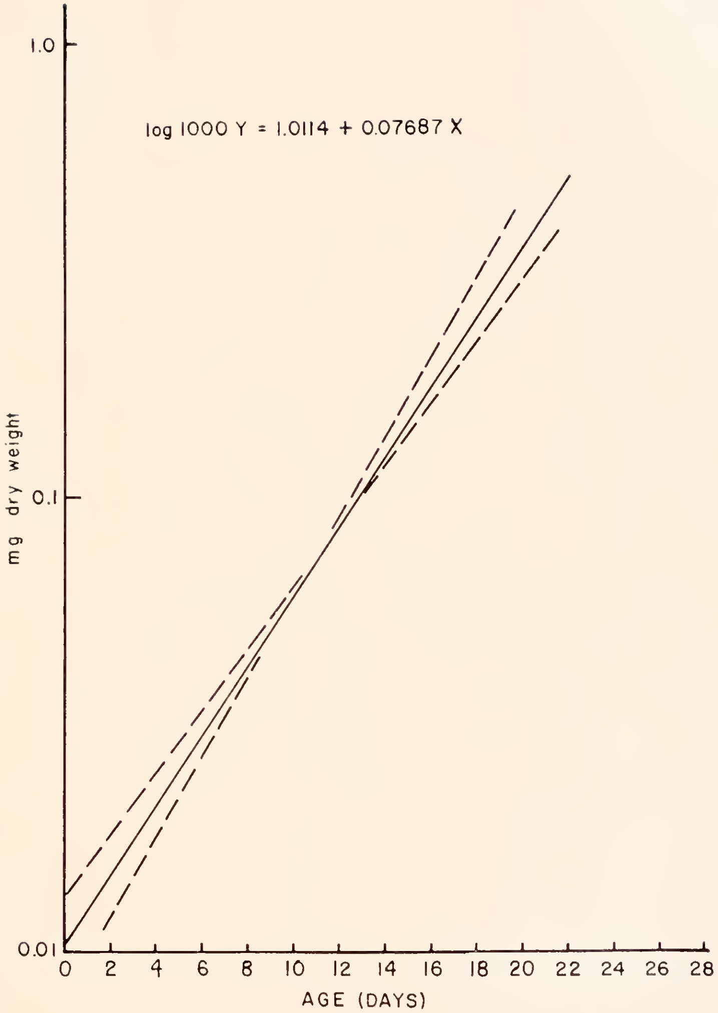

FIGURE 3. Growth expressed as dry weight of the zoea of *Menippe mercenaria* showing 95% confidence limits for the regression coefficient.

morphosis. A decrease in food consumption at molting in crustacean larvae has also been noted by others (Reeve, 1969b; Regnault, 1969). *M. mercenaria* larvae consume up to an average of 91 *Artemia* nauplii per larva per day, which is equivalent to 22 per cent of the dry weight of one animal on day 22. When converted to energy units using a value of 4.538 ± 0.179 calories/mg determined by wet oxidation, individual larvae are seen to consume up to 0.502 calories/day and a total of 7.329 calories from hatching to first juvenile crab.

The rate at which larvae grow and develop was measured in three ways: duration of each larval stage, increase in dry weight, and caloric content. The number of molts recovered per day in survivorship experiments is plotted as a histogram (Fig. 2) with peaks representing the end of each stage. The peaks diminish in

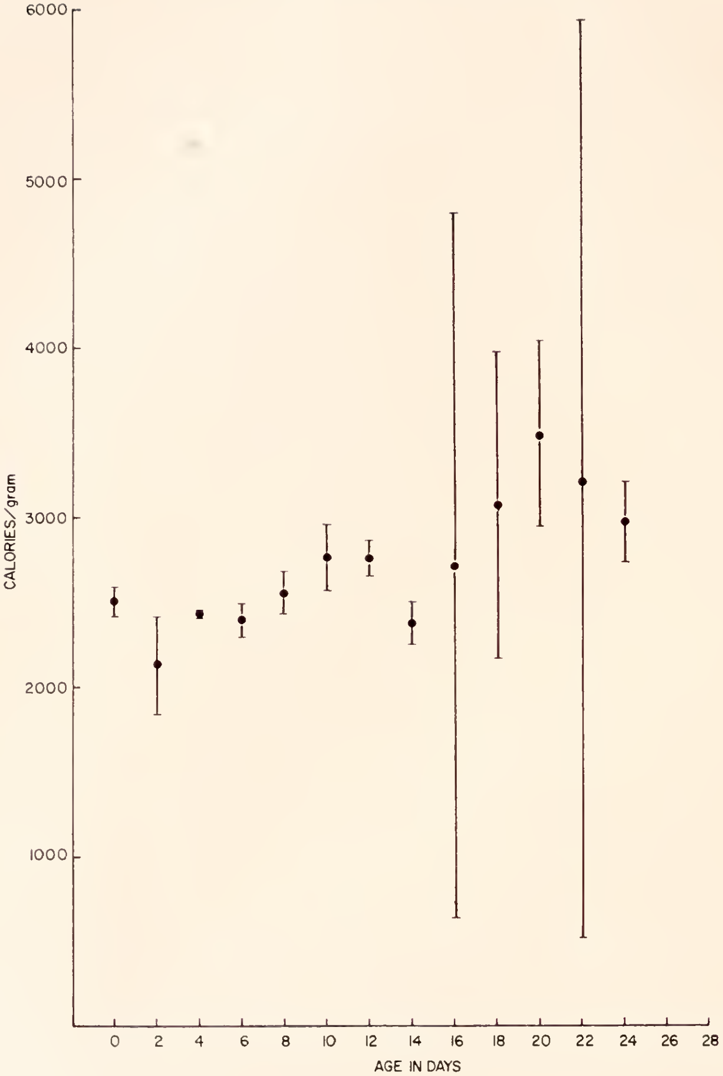

FIGURE 4. Caloric content of *Menippe mercenaria* at two-day intervals from hatching to first juvenile crab, showing the 95% confidence interval.

height as a result of variations in individual developmental time and mortality. For purposes of constructing an energy budget, each stage has been defined as follows: stage 1 from day 0 to 4; stage 2 from day 5 to 8; stage 3 from day 9 to 11; stage 4 from day 12 to 15; stage 5 from day 16 to 20; and megalopa from day 21 to 28.5. The value 28.5 is the mean number of days to first crab determined from the survivorship and molting frequency experiments. It is lower than the value 32.4 given by Ong and Costlow (1970) for *M. mercenaria* under similar conditions of temperature and salinity.

The results of least squares linear regression of log transformed dry weight data with time are shown in Figure 3. Statistical analysis on the fit of the data to the function log 1000Y = 1.0114 + 0.0768X indicates that the slope is significantly different than 0 ($t = 18.129$; $t_{0.01,\ 11} = 3.106$) at the 0.01 probability level. Ninety-five per cent confidence limits for the regression coefficient are plotted (Fig. 3).

Growth is exponential throughout the zoeal stages, representing the exponential phase of the sigmoid growth curve, and levels off at megalopa to an average first crab dry weight of 0.69 mg. Dry weight at hatching is calculated to be 0.0102 mg; by day 20, the beginning of megalopa, the average weight is 0.354 mg, an increase of 35 times over the initial value.

The results of wet oxidation analysis are shown as calories per gram dry weight in Figure 4, with limits for the 0.05 confidence interval. Due to a lack of sufficient replicate samples, the intervals for days 16 to 22 are wide. Values range from a low of 2.129 calories/mg at day 2 to a high of 3.746 calories/mg for newly molted first crabs. Samples of exuvia from each stage were averaged and contain 1.296 calories/mg. In general, the energy content of the larvae increases with age, although it is apparent that a cyclic pattern may exist which could be related to the molting cycle. After hatching, the caloric content drops from an average of 2.503 calories/mg to 2.129 calories/mg. It is assumed that this is due to absorption of yolk material after hatching, which is primarily fat in many marine crustaceans (Pandian and Schumann, 1967) and may contain up to 9.35 calories/mg (Petrusewicz and Macfadyen, 1970). The values obtained are in accordance with other workers using a similar method of analysis (Tsikhon-Lukanina, Soldatova, and Nikolayera, 1968; Tsikhon-Lukanina and Lukasheva, 1970; Soldatova, 1970). They are, however, lower than values obtained for comparable material by the methods of bomb calorimetry.

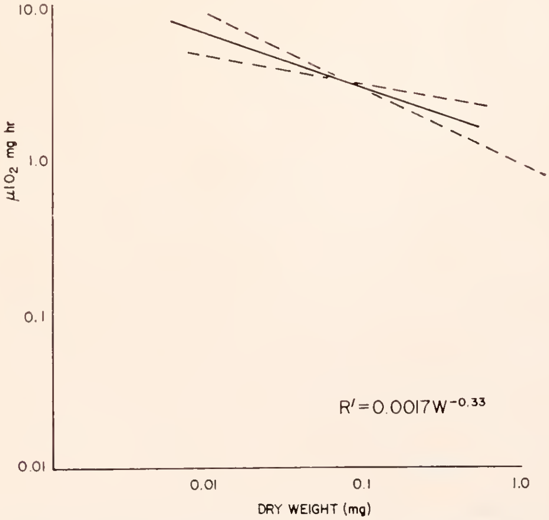

FIGURE 5. Weight specific respiration rate for the zoea of *Menippe mercenaria* with the 95% confidence interval plotted.

TABLE I

Energy budget for each zoeal stage and the megalopa of Menippe mercenaria

Stage	Energy consumed (C) (cal.)	Growth (Pg) (cal.)	Energy of exuvium (E) (cal.)	Respiratory energy expenditure (R) (cal.)	Rejecta (FU) (cal. by diff.)
1	0.211	0.025	0.003	0.060	0.123
2	0.347	0.054	0.005	0.082	0.208
3	0.376	0.085	0.006	0.093	0.192
4	0.838	0.192	0.030	0.188	0.428
5	1.590	0.671	0.057	0.404	0.458
Megalopa	3.980	1.181	0.402	1.105	1.292

Weight specific oxygen consumption, as a measure of energy expended for maintenance, is shown in Figure 5. The data obtained approximate active metabolism rather than basal or resting metabolism since the animals were free to swim within the confines of the respiration chamber. Energy expended on locomotion is therefore taken into account. The energetic cost of feeding and digestion was not included since it was not measured directly and could not be obtained by subtraction. Regression analysis on logarithmically transformed data gives the relationship $R' = 0.0017W^{-0.33}$ where R′ is the weight-specific oxygen consumption and W is the dry weight of the animal. The alternate equation, $R = 0.0017W^{0.67}$, where R is the oxygen consumption per unit time for one organism, fits the theoretical assumption that respiration rate is proportional to the two thirds power of body weight. Statistical analysis on the data indicates that the regression coefficient is significantly different than 0 ($t = 4.714$; $t_{0.01,\ 16} = 2.921$). Ninety-five per cent confidence limits for the regression coefficient are plotted. Richman's (1958) values for *Daphnia pulex* are 0.0014 and 0.881 for a and b, respectively, corresponding to 0.0017 and −0.67, above. Clutter and Theilacker (1971) give a as 2.0 and b as 0.62 to 0.68 in *Metamysidopsis elongata*. Generally, b is between 0.67 and 1.00 for Crustacea (Wolvekamp and Waterman, 1960). The results of this study compare well with similar studies on oxygen consumption in Crustacea.

DISCUSSION

The data presented above are used in the calculation of an energy budget and various energetic efficiencies for the larvae of *Menippe mercenaria*. Table I shows the partitioning of consumed energy among the various life processes for each zoeal stage and the megalopa. In the second column, the energy consumed represents the total calories ingested per larva for each stage. Consumption increases over ten times from stage one to megalopa while the average dry weight increases thirty-five times, indicating an increase in the efficiency with which energy is utilized for growth. Energy expended on growth is equal to the sum of the calculated weight gain per day multiplied by the caloric content per unit weight for the corresponding day. The amount of energy channeled into growth is greater for each succeeding stage. When considered on a daily basis, however, energy of growth increases through stage 5 and levels off at megalopa, similar to growth as dry weight. Production of exuvia represents a loss to the organism at each successive molt. While this loss is small compared to growth for zoea, it represents approximately 26 per cent of total production at megalopa (Table I).

Respiratory energy expenditure is the caloric equivalent of oxygen consumption for an average RQ of 0.82 (Brody, 1945), which corresponds to 4.825×10^{-3} calories/μl O_2 consumed (Table I). The maximum error obtained by estimating RQ rather than experimentally determining it is only about 3.5 per cent when oxygen consumption rather than carbon dioxide production is used (Brody, 1945). This is well within the limits of experimental error in metabolic measurements. More energy is channeled into respiration with succeeding stages and increasing size. When respiratory energy expenditure is compared with that utilized for production (growth and exuvia), the reverse is seen. A larger proportion of the assimilated calories are used for respiration at stage 1; respiration and production equalize at stage 3, while energy going into production exceeds that for respiration by stage 5, when growth is at its peak. Due to the increased mass of the megalopal exuvium, respiratory energy expenditure exceeds production during megalopa.

Rejecta as used here (Table I) is the difference between energy consumed and assimilated (sum of growth, energy of exuvia, and respiration). It represents energy not utilized by the organism, a possible example being the exoskeleton of the *Artemia* nauplii. It includes fecal and urine production. Due to the size of the animals used in this study, it was not feasible to obtain the values directly.

From the energy budget presented above, efficiencies are calculated which give the proportions of energy utilized for each process. Assimilation efficiency, gross growth efficiency, and net growth efficiency are the most relevant to larval stages where development of reproductive tissue is negligible. Per cent assimilation (Table II) is the ratio between assimilated calories and calories consumed and represents the proportion of the energy consumed by the organism which is actually available for growth and respiration. For *Menippe mercenaria* larvae it increases from 41.70 per cent during stage 1 to 85.43 per cent at megalopa. The values obtained may be biased since only whole *Artemia* nauplii were counted in determining feeding rate; however, no recognizable fragments of *Artemia* were ever seen.

Assimilation efficiency is highly variable depending on the species, the age of the organism, the type of food and its concentration, internal physiological factors, the environmental conditions, and the way in which it is measured (Petrusewicz and Macfadyen, 1970). For example, excretion is generally considered negligible

TABLE II

Energetic efficiencies of zoeal stage and the megalopa of Menippe mercenaria

Stage	Percent assimilation (A/C)	Gross growth efficiency (P_g/C)	Net growth efficiency (P_g/A)
1	41.70	11.84	28.41
2	40.40	15.56	38.30
3	48.91	22.61	46.19
4	48.93	22.91	46.83
5	71.19	42.20	59.93
Megalopa	85.43	29.67	43.94

in energy studies, but it has been shown to be as much as 36 per cent of the assimilated calories (Hargrave, 1971). When assimilation is measured as consumption minus egestion, excretion is ignored. The indirect methods of this study consider excretion and egestion together. While assimilation efficiency is important to the individual organism, particularly in nutritional studies where optimization of feeding and growth are desired (*i.e.*, agriculture or mariculture), comparison of data for different species may be of little value. For example, values reported range from 6.6 per cent for pre-adult *Daphnia pulex* (Richman, 1958) to 100 per cent for the wolf spider (Edgar, 1971) and from 15 to 99 per cent for *Calanus finmarchicus* (Marshall and Orr, 1955).

Gross growth efficiency is defined as the proportion of consumed energy utilized for growth (P_g), while net growth efficiency is the ratio between growth (P_g) and assimilated energy. The net growth efficiency is always higher than the gross growth efficiency, with one exception—the case where assimilation is 100 per cent.

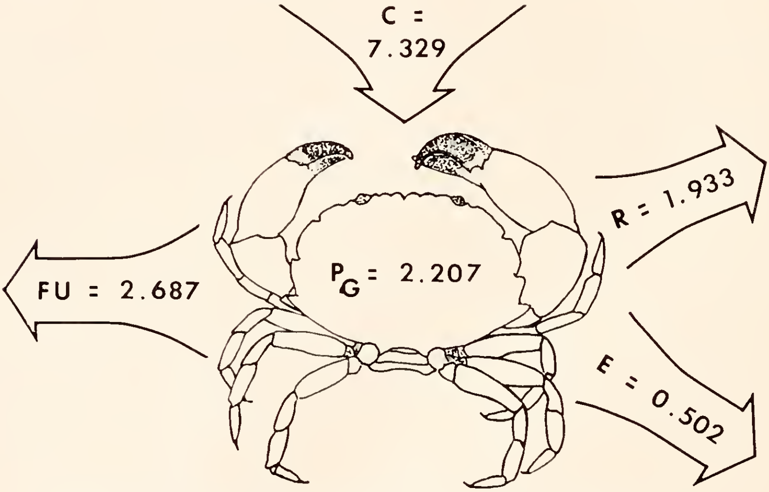

FIGURE 6. Cumulative energy budget from time of hatching to first juvenile crab showing all energy sources and sinks.

In this study, exuvia (E) were not included in the growth efficiency calculations since they represent a loss of energy to the organism. The larvae of *M. mercenaria* utilize energy most efficiently for growth during the third, fourth, and fifth zoeal stages. With decreased growth rate during megalopa, the efficiency with which energy is utilized for growth also decreases. Reeve (1969b) found growth efficiency to decrease from 70 to 35 per cent over larval development in *Palaemon serratus*. However, his work was based on dry weight values alone, so changes in caloric content of the larvae with time were not taken into account. The growth efficiencies obtained compare with literature values for developing stages of *Daphnia pulex* (Richman, 1958) and *Calanus hyperboreus* (Conover, 1964) but are considerably higher than those of young isopods (Tsikhon-Lukanina and Lukasheva, 1970).

A cumulative energy budget from hatching to first crab is presented in Figure 6. It represents the total amount of energy utilized during larval development in calories. Of the 7.329 calories consumed (C), 2.207 calories are used for growth (P_g), 1.933 calories for respiratory energy expenditure (R), 0.502 calories for production of exuvia (E), and 2.687 calories are eliminated through egestion and excretion (FU). The cumulative assimilation efficiency is 63.33 per cent, gross growth efficiency is 30.11 per cent, and net growth efficiency is 47.54 per cent. Conover (1964, 1966) suggests an assimilation efficiency for crustaceans of at least 60 per cent and perhaps greater. Reeve (1969b) found a cumulative gross growth efficiency of 35 per cent for *Palaemon serratus* fed *Artemia* nauplii. Both compare with the results obtained in this experiment.

In general, maintenance of vital life processes constitutes the major energy expenditure for invertebrate populations. Production (growth and reproduction) accounts for only 10 to 20 per cent of the total (Phillipson, 1966). However, when only a portion of the life cycle is considered, energy utilized for growth may equal or exceed that used for maintenance. Richman (1958) found that pre-adult *Daphnia pulex* expend 55.36 to 58.64 per cent of assimilated energy on growth; and adults, at the time of reproduction, utilize 52.08 to 70.46 per cent of the energy assimilated on production of young. The copepod *Calanus hyperboreus* exhibits a net growth efficiency of up to 89 per cent by storing fat during the short-lived phytoplankton blooms of the Arctic summer (Conover, 1964). In the fifth zoeal stage, *Menippe mercenaria*, as shown in this study, can convert up to 59.93 per cent of the energy it assimilates into new body tissue. When production of exuvia is taken into account, this increases to 64.31 per cent. The ability to channel large amounts of energy into production presents an obvious advantage to the organisms. Arctic copepods can store enough energy to sustain them through much of the winter. Larvae of benthic invertebrates, as *Menippe mercenaria*, can take advantage of the food supply available in the plankton to maximize growth and possibly shorten developmental time before settling to the bottom.

The construction of an energy budget is important both from a nutritional and ecological point of view. Unfortunately, the data must be obtained under laboratory conditions which, at best, can only approximate the natural state, making application to the field difficult (Petrusewicz and Macfadyen, 1970). Some energy budget parameters are difficult to measure, particularly on very small organisms. Feeding rate and growth are the easiest parameters to measure with minimal disturbance to the organisms. Consequently, energy consumed and energy of growth are the most reliable data in this study. Maintenance energy data are the least accurate of the parameters measured since the animals were confined to small chambers and unfed during the respiration experiments. Had the animals been fed, it would have been impossible to account for the oxygen demand of fecal matter produced during the experiments. The methods used were intended to give the most uniform results possible.

This study is the most complete one to date on energetics of marine crustacean larvae. Analysis of the data by larval stage, as well as cumulatively from hatching to first crab, shows significant changes during development in growth, efficiency of food utilization for growth, and assimilation efficiency. The limited scope of the study (single temperature regime, single food source at one density, and single salinity investigated) prevents construction of an ecological energy budget. An extension of this study to investigate the effects of environmental parameters on the energetics of the species would allow a more complete and natural picture.

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SUMMARY

1. An energy budget was constructed for *Menippe mercenaria* from hatching to first juvenile crab when fed newly-hatched *Artemia* nauplii.

2. Larvae of *M. mercenaria* grow exponentially through the zoeal stages. Growth rate decreases during megalopa.

3. Consumption reaches its peak during megalopa where one individual may consume 91 *Artemia* nauplii/day. Feeding rate decreases prior to molting to first crab stage.

4. The caloric content per unit dry weight increases from 2.503 cal/mg at hatching to 3.746 cal/mg at first juvenile crab. Molts contain 1.296 cal/mg.

5. Respiration by zoea is proportional to the two thirds power of body weight and is described by the function $R = 0.0017W^{0.67}$.

6. Over the period of larval development, 7.329 calories are consumed, 2.207 calories are used for growth, 0.502 calories are lost to production of exuvia, 1.933 calories are expended for maintenance, and 2.687 calories are lost as rejecta.

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ANION AND CATION REQUIREMENTS FOR GLUCOSE AND METHIONINE ACCUMULATION BY *HYMENOLEPIS DIMINUTA* (CESTODA)[1]

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The Na^+- or ion-gradient hypothesis (see Schultz and Curran, 1970, for a review) postulates that the energy necessary for the intracellular accumulation of solutes against a concentration difference is obtained, ultimately, from the maintenance of a Na^+, and possibly K^+, difference across the cell membrane. In addition to evidence that such a mechanism is functional in glucose and amino acid influx and accumulation in some vertebrate tissues (Schultz and Curran, 1970), there is ample evidence that such systems also function in glucose influx in tapeworms. Glucose influxes in *Taenia (Hydatigera) taeniaeformis* larvae and adults, *Hymenolepis diminuta* larvae and adults, *Calliobothrium verticillatum* adults, and *Taenia crassiceps* larvae are Na^+-sensitive (von Brand and Gibbs, 1966; Arme, Middleton and Scott, 1973; Dike and Read, 1971; Fisher and Read, 1971; Pappas, Uglem and Read, 1973a, respectively). Glucose and Na^+ movements into *C. verticillatum* are coupled, and the kinetics of glucose and Na^+ fluxes in this species suggest that the mechanisms of Na^+-coupled glucose influx in tapeworms and vertebrate tissues are generally similar (Pappas and Read, 1972).

On the other hand, the influxes of phenylalanine and methionine in *T. crassiceps* larvae and methionine in *H. diminuta* are insensitive to the external Na^+ concentration (Pappas, Uglem and Read, 1973b, Read, Rothman and Simmons, 1963, respectively), although these amino acids are accumulated by the worms in the presence of Na^+. Moreover, *T. crassiceps* larvae accumulate phenylalanine and methionine in Na^+-free media (Pappas *et al.*, 1973b). This raises the question of whether ions and/or ion differences (gradients) are related to amino acid influx and accumulation in tapeworms. Also, although glucose influx in several tapeworm species is known to be Na^+-sensitive and/or Na^+-coupled, it is not known whether glucose accumulation is Na^+-dependent. This paper reports an experimental examination of ion requirements for glucose and amino acid influx and accumulation in the tapeworm, *Hymenolepis diminuta*.

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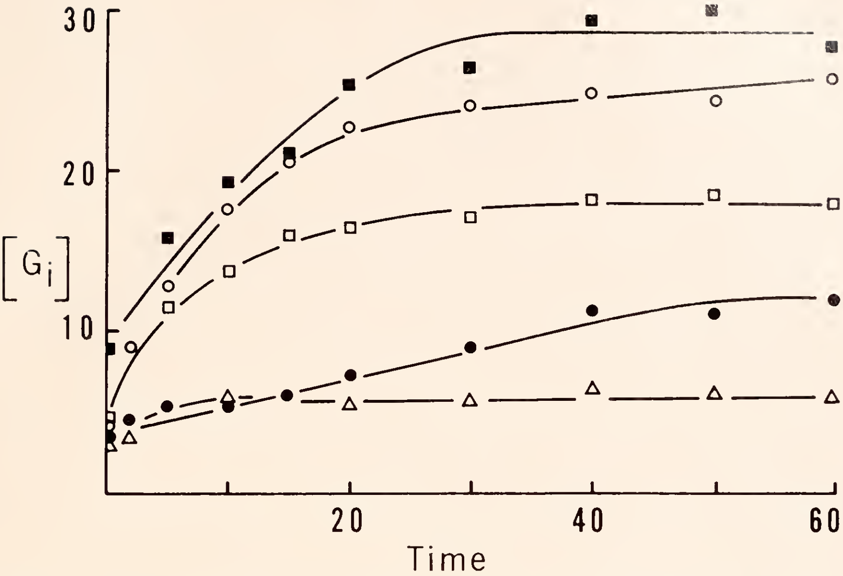

FIGURE 1. The internal glucose concentration ([G_i], µmoles glucose/ml worm water) of *Hymenolepis diminuta* incubated in 5 mM glucose in KRT, or various ion-substituted KRT solutions, versus time of incubation (min). Closed circles equal control (worms incubated in KRT only); open circles equal worms incubated in glucose in KRT; open triangles equal worms incubated in glucose in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in glucose in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3COO^- as the replacement anion. Each point is the mean of 3 replicates.

MATERIALS AND METHODS

Specimens of *Hymenolepis diminuta* from 10-day-old, 30-worm infections of young male Sprague-Dawley rats (Holtzman Co.) were used in all experiments. After removal from the host, worms were washed in several changes of a Krebs-Ringer solution containing 25 mM tris(hydroxymethyl)-aminomethane-maleate buffer at pH 7.4 (KRT of Read *et al.*, 1963), and randomized into groups of 5 worms (approximately 200 mg wet wt). Prior to incubation, each group of worms was preincubated in 10 ml of fresh KRT (or the appropriate ion-substituted KRT) for 30 min. All preincubations and incubations were carried out at 37° C.

In L-methionine and D-glucose accumulation studies, the worms were removed from the preincubation medium, blotted dry, weighed (± 0.5 mg), and incubated in 10 ml of KRT (or the appropriate ion-substituted KRT) containing either L-methionine or D-glucose. After intervals of time indicated in various experiments, the worms were removed, rinsed rapidly in 3 changes of KRT, blotted dry, weighed (± 0.5 mg), and extracted overnight in 70% ethanol. Glucose in the ethanol extracts and incubation media were determined using the glucose oxidase method (Glucostat Special, Worthington Biochemicals), and methionine in the ethanol extracts (following partitioning against acidified chloroform) and incubation media was determined by the method of Horn, Jones and Blum (1946). Glucose and methionine concentrations in the worms were calculated in terms of μmoles solutes/ml worm water at the end of the incubation period. Following ethanol extraction, the worms were digested in 30% KOH and the 50% ethanol precipitable carbohydrate (glycogen) was determined by the method of Dubois, Giles, Hamilton, Rebers and Smith (1956).

To measure short term glucose and methionine influx rates, groups of worms were incubated in 5 ml of KRT (or the appropriate ion-substituted KRT) containing 0.25 μCi/ml of D-glucose-^{14}C(U) or L-methionine-^{14}C(methyl) (New England Nuclear). After a 2 min incubation, the worms were removed, rinsed rapidly in 3 changes of KRT, blotted dry and extracted overnight in 2 ml of 70% ethanol. Radioactivity in 1 ml aliquots of the ethanol extracts was determined with a Nuclear-Chicago gas-flow counter. The worms were dried overnight at 95° C and weighed.

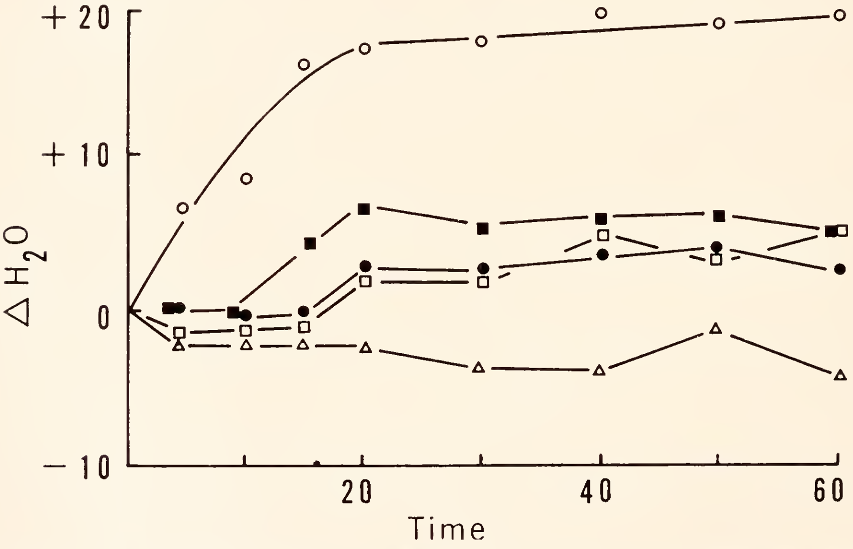

FIGURE 2. Percentage change in the worm water (ΔH_2O) of *Hymenolepis diminuta* incubated in 5 mM glucose in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Symbols as in Figure 1. Each point is the mean of 3 replicates.

RESULTS

The internal glucose concentration ([G$_i$]) of *H. diminuta* incubated in KRT for 60 min rose from an initial 4 mM to 12 mM, in terms of worm water. During this period, there was a net water influx representing a 5% increase in worm water, and a net decrease in worm glycogen. When incubated in 5 mM glucose in KRT for 60 min, the [G$_i$] rose from 4.2 mM to 25.5 mM, and was accompanied by a 20% increase in worm water; under these conditions worm glycogen increased (Figs. 1 and 2, Table I). Glucose in the incubation medium decreased from 5.0 mM to 3.8 mM during the 60 min incubation. However, of the 12 μmoles removed from the incubation medium (10 ml at 1.2 μmoles/ml) by worms, only 5 μmoles of free glucose was recovered in the ethanol extracts indicating that 60% of the absorbed glucose was metabolized.

When worms were incubated in 5 mM glucose in Na^+-free KRT (with choline as the replacement cation) for 60 min, the $[G_i]$ rose from 3.8 mM to 6 mM, while only 2–5% of the initial worm water was lost. Glucose in the incubation media increased from 4.98 mM to 5.25 mM after 60 min, and worm glycogen decreased (Figs. 1 and 2, Table I).

The steady state free glucose concentration of worms incubated in Cl^--free KRT (Cl^- replaced with CH_3COO^- or NO_3^-) with 5 mM glucose rose significantly above the glucose concentration of the media. With NO_3^- as the replacement anion the $[G_i]$ attained was approximately equal to that attained by worms incubated in glucose in KRT (27 mM and 25.5 mM, respectively), while in media with CH_3COO^- as the replacement anion the steady state $[G_i]$ was reduced (18 mM). There was a small net influx of water (5–6% of the original worm water) in worms incubated in glucose in Cl^--free KRT. Worms incubated in Cl^--free KRT with NO_3^- as the replacement anion removed a slightly greater amount of glucose than did worms in media with CH_3COO^- as the replacement anion; in both experiments 55–60% of the glucose removed from the media in 60 min was not recovered in the ethanol extracts, *i.e.*, it was metabolized. The glycogen levels of worms incubated in glucose in Cl^--free KRT increased (Figs. 1 and 2, Table I).

TABLE I

Glycogen levels (mg/g wet wt) of Hymenolepis diminuta *before (control) and after a 60 min* in vitro *incubation in various media. Values are listed as means of 3 replicates ± S.E.*

Incubation medium	Glycogen
A. Control (none)	92.6 ± 5.6*
B. KRT	85.4 ± 4.8
C. 5 mM glucose in KRT	100.8 ± 3.9
D. 5 mM glucose in Na^+-free KRT**	86.4 ± 4.1
E. 5 mM glucose in Cl^--free KRT***	97.6 ± 4.6
F. 2 mM methionine in KRT	87.2 ± 4.1
G. 2 mM methionine in Na^+-free KRT**	87.8 ± 5.0
H. 2 mM methionine in Cl^--free KRT***	88.0 ± 4.5

* Statistical differences were determined using a one-way analysis of variance and Student-Newman-Keuls *a posteriori* test. The means were grouped as follows: (B, D, F, G, H) (A) (C, E); Each group of means is different from the other two groups at the 95% level of significance.

** Choline as the replacement cation.

*** CH_3COO^- as the replacement anion.

The method of Horn *et al.* (1946) was not sensitive enough to detect the small free methionine pool known to exist in *H. diminuta* (J. E. Simmons, Jr., unpublished), nor did this method reveal any changes in extractable methionine when worms were incubated in KRT for 60 min. However, when worms were incubated in 2 mM methionine in KRT, Na^+-free KRT (with choline as the replacement cation), or Cl^--free KRT (with either NO_3^- or CH_3COO^- as the replacement anion), increases in the free methionine of worms were easily detected, and worms were found to accumulate methionine in all media (Fig. 3). The internal methio-

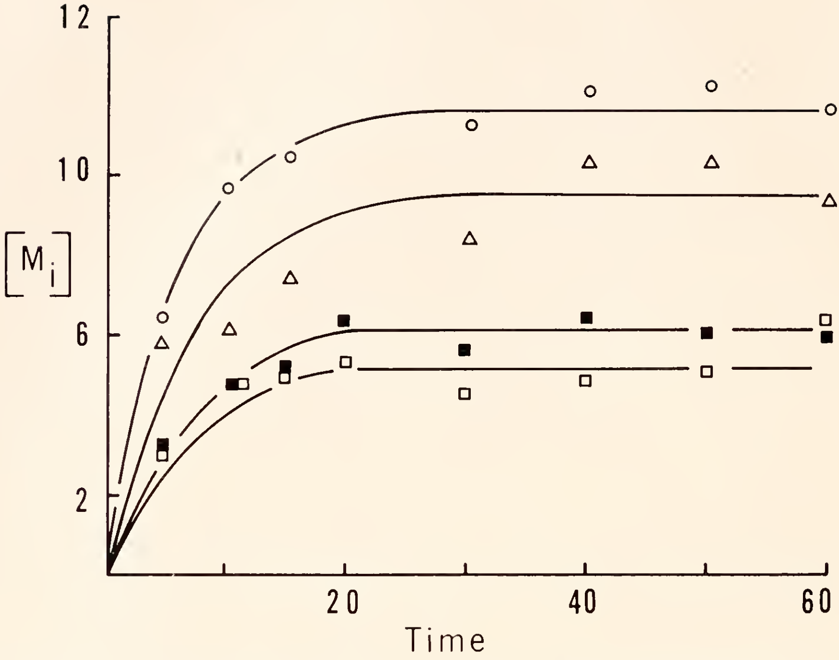

FIGURE 3. The internal methionine concentration ($[M_i]$, μmoles methionine/ml worm water) of *Hymenolepis diminuta* incubated in 2 mM methionine in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Open circles equal worms incubated in methionine in KRT; open triangles equal worms incubated in methionine in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in methionine in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3COO^- as the replacement anion. Each point is the mean of 3 replicates.

nine concentration ($[M_i]$) at a steady state was about 11.5 mM and 9.5 mM for worms incubated in 2 mM methionine in KRT and Na^+-free KRT, respectively. Although the steady state $[M_i]$ of worms in Na^+-free media appeared to be slightly depressed, the values for the $[M_i]$ of worms at 40, 50, and 60 min incubation in KRT and Na^+-free KRT were not significantly different ($P > 0.05$ by Student's *t* test). However, the steady state $[M_i]$ of worms incubated in 2 mM methionine in Cl^--free KRT (with either replacement anion) was significantly lower than that of worms incubated in methionine in KRT. $[M_i]$ values of worms incubated in NO_3^- and CH_3COO^--substituted media were not significantly different.

In KRT, there was a net water influx with about 5% increase in worm water; however, with the addition of 2 mM methionine, water effluxed from the worms regardless of the ionic composition of the media (Fig. 4). Water efflux from worms incubated in methionine in KRT or Na^+-free KRT was less than 5% of the original worm water, but in Cl^--free KRT, the water efflux rose as high as 10%. Glycogen levels of worms decreased when incubated in the absence of glucose (Table I). Methionine in the incubation media was not depleted to a significant extent in any experiment.

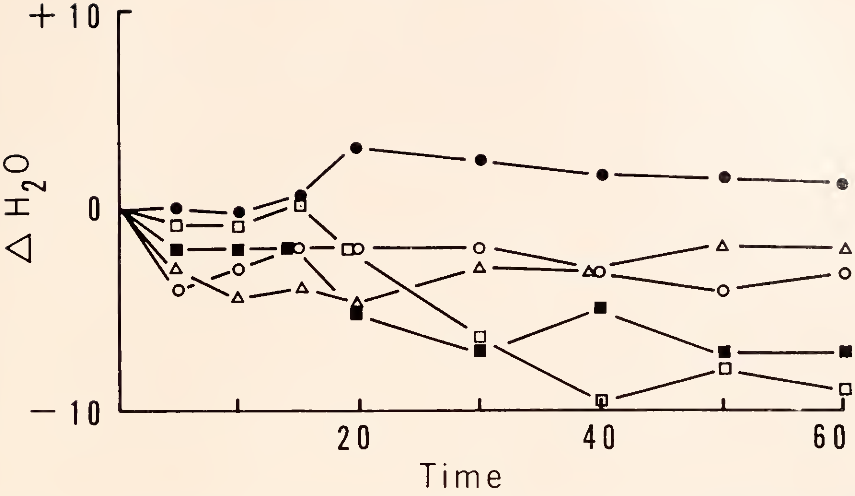

FIGURE 4. Percentage change in the worm water (ΔH_2O) of *Hymenolepis diminuta* incubated in 2 mM methionine in KRT, or various ion-substituted KRT solutions, *versus* time of incubation (min). Closed circles equal control (worms incubated in KRT only); open circles equal worms incubated in methionine in KRT; open triangles equal worms incubated in methionine in Na^+-free KRT with choline as the replacement cation; closed squares equal worms incubated in methionine in Cl^--free KRT with NO_3^- as the replacement anion; open squares equal worms incubated in Cl^--free KRT with CH_3OO^- as the replacement anion. Each point is the mean of 3 replicates.

Glucose influx rates in *H. diminuta* were extremely sensitive to the external Na^+ concentration. Glucose influx was inhibited 96% in Na^+-free media (with choline as the replacement cation). Glucose influx rates were less sensitive to external Cl^- concentrations, and significant inhibition of glucose influx was obtained only at very low Cl^- concentrations (Table II). Methionine influx rates were unaffected by deletion of Na^+ or Cl^- from the media (Table III).

TABLE II

The effect of Na^+ and Cl^- deletion on the influx (μmoles/g ethanol-extracted dry wt/2 min) of ^{14}C-glucose in Hymenolepis diminuta. *Values are listed as means of 3 replicates ± S.E.*

$[Na^+]$, mM	Choline as replacement cation	$[Cl^-]$, mM	CH_3COO^- as replacement anion	NO_3^- as replacement anion
0	0.05 ± 0.006*	0	8.61 ± 1.46**	7.38 ± 0.42*
5	0.65 ± 0.06	5	12.88 ± 0.96	—
10	1.26 ± 0.06	10	11.00 ± 0.73	7.01 ± 0.36
15	2.91 ± 0.16	25	13.89 ± 0.93	8.04 ± 0.29
25	3.96 ± 0.18	50	13.83 ± 1.22	8.31 ± 0.58
50	6.06 ± 0.23	75	14.39 ± 0.78	9.27 ± 0.59
75	7.86 ± 0.39	100	12.73 ± 0.88	9.78 ± 0.22
100	9.68 ± 0.67	130	14.58 ± 1.79	11.94 ± 0.18
154	11.15 ± 0.59			

* Glucose concentration = 1 mM
** Glucose concentration = 2.5 mM.

TABLE III

The effect of Na^+ and Cl^- deletion on the influx (μmoles/g ethanol extracted dry wt/2 min) of ^{14}C-methionine in Hymenolepis diminuta. *Values are listed as means of 3 replicates ± S.E.*

$[Na^+]$, mM	Choline replacement cation	$[Cl^-]$, mM	CH_3COO^- as replacement anion	NO_3^- as replacement anion
0	14.34 ± 0.89*	0	11.09 ± 0.22**	11.18 ± 0.58**
5	12.88 ± 0.38	5	10.80 ± 0.28	—
10	15.35 ± 0.60	10	—	10.98 ± 0.61
25	13.30 ± 0.40	15	11.52 ± 0.76	—
50	13.17 ± 0.78	25	11.83 ± 0.39	11.86 ± 0.51
100	16.30 ± 0.83	50	11.79 ± 0.54	12.13 ± 0.81
154	14.59 ± 0.64	75	12.26 ± 0.62	11.08 ± 0.72
		100	12.39 ± 1.06	11.82 ± 0.71
		130	12.00 ± 0.67	11.61 ± 0.68

* Methionine concentration = 2.5 mM.
** Methionine concentration = 2 mM.

DISCUSSION

Previous studies have shown that some tapeworms have Na^+-dependent glucose transport systems (see introduction), and *H. diminuta* is no exception. As previously shown by Dike and Read (1971), glucose influx in *H. diminuta* is inhibited almost completely in Na^+-free media, and more recent studies have shown that glucose and Na^+ fluxes in *H. diminuta* are coupled (Read, Stewart and Pappas, unpublished). It is, therefore, not surprising to find that *H. diminuta* does not accumulate glucose in Na^+-free media. Apparently, the lack of glucose accumulation is related to the Na^+-dependence of glucose fluxes, with the energy-coupling device for glucose accumulation being maintenance of a Na^+ gradient across the tapeworm tegument. It might be argued that glucose is not accumulated in Na^+-free media because the worms are not able to absorb glucose which would subsequently be used for energy production (Read, 1967); however, it should be noted that the worms produce glucose during incubation in Na^+-free media (*via* glycogenolysis), and also that worms accumulate methionine in the absence of an external energy source (*i.e.*, glucose). Therefore, the inhibition of glucose accumulation in Na^+-free media because of a lack of glucose as an energy source seems an improbable explanation.

When incubated in KRT only, the $[G_i]$ of worms increases significantly while glycogen levels decrease. (There is little doubt that this increase in tissue glucose is a direct consequence of glycogenolysis, since glucose is the only glycogenic monosaccharide [Read, 1967], and since worms degrade glycogen when incubated in the absence of external glucose.) We might expect similar results from worms incubated in glucose in Na^+-free KRT since, under these conditions, no glucose enters the worms. Although glycogen levels in worms incubated in glucose in Na^+-free KRT decrease, there is no concomitant increase in tissue glucose. This apparent discrepancy might be explained by the observation that the glucose concentration of the Na^+-free media increases during incubation with worms, the additional glucose coming from glycogenolysis and subsequent glucose efflux from the worms. Such a "trans" effect from a reversed Na^+ difference (gradient) also occurs in the tapeworm *C. verticillatum*, as shown by Pappas and Read (1972). These data support the hypothesis that glucose fluxes in *H. diminuta* are controlled, in large part, by the prevailing Na^+ difference.

Although pigeon erythrocytes contain at least 3 specific amino acid transport systems, one of which is sensitive to both Na^+ and Cl^- (Imler and Vidaver, 1972), no animal cell, to our knowledge, has been shown to contain a single glucose transport system which is sensitive to both anions and cations. *H. diminuta* may be the exception since glucose influx is inhibited in both Na^+- and Cl^--free media. No data are available which suggest a possible mechanism for this Cl^--sensitivity. However, since replacement of Cl^- with NO_3^- or CH_3COO^- yields similar results (38% and 41% inhibition, respectively) and since methionine influx is not inhibited in Cl^--free media, it appears that the effects of Cl^- deletion are rather specific. Although these data are suggestive of an anion requirement for glucose influx, further studies are needed to clarify this point.

H. diminuta accumulated glucose in Cl^--free media, but the steady state $[G_i]$ of worms is reduced when CH_3COO^- is used to replace deleted Cl^-. Since replacement of Cl^- with either NO_3^- or CH_3COO^- has similar results on glucose influx, we may speculate that replacement of Cl^- with CH_3COO^- increases the efflux of glucose from *H. diminuta*. This should be examined experimentally. However, it is clear the glucose influx and accumulation in *H. diminuta* do not display an absolute requirement for Cl^-, as they do for Na^+.

As originally reported by Read *et al.* (1963), methionine influx in *H. diminuta* is not Na^+-sensitive. Similar results were obtained by Uglem (unpublished) with lysine, glutamate and leucine influxes in *H. diminuta*, and Pappas *et al.* (1973b) showed that methionine, phenylalanine and arginine influxes into *T. crassiceps* larvae are not Na^+-sensitive. Methionine accumulation by *H. diminuta* is also Na^+-insensitive, as is methionine and phenylalanine accumulation by *T. crassiceps* larvae (Pappas *et al.*, 1973b). Unlike glucose influx and accumulation, methionine influx and accumulation in *H. diminuta* and *T. crassiceps* larvae are not dependent upon Na^+ in the external medium. A similar situation may exist in the Acanthocephala, since Uglem and Read (1973) found that leucine is accumulated by *Moniliformis dubius* in Na^+-free media.

Deletion of Cl^- and its replacement with NO_3^- or CH_3COO^- has no effect on methionine influx, however, the steady state $[M_i]$ of worms incubated in 2 mM methionine in Cl^--free media is reduced, compared to worms incubated with methionine in KRT. It appears that either amino acid influx and accumulation are not Cl^--sensitive, or that both NO_3^- and CH_3COO^- will replace Cl^- if this system is anion sensitive. In pigeon erythrocytes, glycine influx occurs through a system which cotransports Na^+ but which also must bind an anion for amino acid translocation. F^-, NO_3^-, HCO_2^-, SCN^-, and I^- will replace Cl^- as the anion cofactor, although they are less efficient than Cl^-, while CH_3COO^-, $CH_3CH_2(OH)COO^-$ (lactate), and larger anions will not replace Cl^- (Imler and Vidaver, 1972). Methionine influx in *H. diminuta* apparently occurs through a system quite different than that found in other anion and/or cation-requiring systems.

As stated above, the data regarding glucose influx and accumulation are consistent with Na^+-gradient hypothesis as originally proposed by Crane (1962, 1965).

However, methionine influx and accumulation do not depend upon the maintenance of either a Na^+ or Cl^- difference across the cell membrane. That is, the maintenance of an ion difference does not appear to be involved in energy-coupling for methionine accumulation, as the data suggest it to be for glucose accumulation. Therefore, we may infer that the energy necessary for methionine accumulation must be derived from some other source. Several studies have shown that the potential energy available from the Na^+ difference across cell membranes is sufficient to account for amino acid and glucose accumulation in some mammalian intestinal preparations and pigeon erythrocytes, however, in other systems (chick intestine and ascites tumor cells), the Na^+- gradient does not appear to provide sufficient potential energy for solute influx and accumulation (see Gibb and Ebby, 1972, and Johnstone, 1972, and references therein), and it has been suggested that additional energy is derived from the maintenance of K^+ difference or from ATP directly. No studies are available which deal with energy requirements of solute influx and accumulation in *H. diminuta,* but it appears that the energy for methionine accumulation must be derived from some source other than Na^+ or Cl^- concentration differences across the cell membranes.

For the preceding discussion we have made the tenuous, but necessary, assumption that water associated with *H. diminuta* (as determined from [wet wt]–[dry wt]) exists in a free state within this tapeworm and that solutes are in "true solution." In a recent symposium (Hazelwood, 1973) the physical and chemical state of water and solutes within living cells was discussed at length and, from data presented in these papers, it is apparent that neither of our assumptions are completely valid. However, at the present time an accurate estimation of "bound" *versus* "unbound" water and solutes cannot be made. This situation is complicated by the water fluxes associated with solute accumulation by *H. diminuta.* Although these fluxes are generally small ($< 5\%$ of the original worm water), glucose accumulation by *H. diminuta* is accompanied by a 20% increase in worm water, and methionine accumulation in Cl^--free media is accompanied by a 10% loss of worm water. Like the physico-chemical state of water already present in the worm tissues, the true state of the water influxing during glucose accumulation is uncertain. In addition, many tapeworm species, including *H. diminuta*, appear to have the capacity to regulate their water volume (Read, Douglas and Simmons, 1959; Read and Simmons, 1963; Smyth, 1969; DeRycke, 1972), although the exact mechanism of this water regulation is unknown. It is apparent, therefore, that additional studies are necessary, not only to determine the physicochemical state of solutes and water within *H. diminuta,* but also to determine the osmoregulatory capabilities of tapeworms and the significance of the water fluxes associated with solute accumulation.

SUMMARY

When incubated in 5 mM glucose in a buffered Krebs-Ringer saline for 60 min, *Hymenolepis diminuta* accumulated glucose to a steady state concentration of 25.5 mM; in Cl^--free saline, with either NO_3^- or CH_3COO^- as the replacement anion, glucose was accumulated by worms. *H. diminuta* incubated in 5 mM glucose in Na^+-free media, with choline as the replacement cation, did not accumulate glucose. Short term glucose influx (2 min) in *H. diminuta* was inhibited 96% in Na^+-free

media, and approximately 40% in Cl^--free media. The data support the hypothesis that glucose influx and accumulation in *H. diminuta* occurs through a Na^+-dependent system similar to that proposed by the "ion-gradient hypothesis." The data suggest further that glucose influx in *H. diminuta* may be anion-sensitive as well.

H. diminuta accumulated methionine when incubated in 2 mM methionine in normal saline, Na^+-free media, or Cl^--free media, and short term methionine influx (2 min) in *H. diminuta* was unaffected by Na^+ or Cl^- deletion. Methionine influx and accumulation are not dependent on anion or cation differences (gradients) across the cell membranes and the energy necessary for solute accumulation must be derived from some source other than maintenance of these ion-differences.

Solute accumulation in *H. diminuta* was accompanied by significant water fluxes in some instances. Since the physical state of water and solutes within worms and the worms osmoregulatory capacities are unknown, the significance of these water fluxes is uncertain.

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LARVAL DEVELOPMENT OF *PAGURUS LONGICARPUS* SAY REARED IN THE LABORATORY. V. EFFECT OF DIET ON SURVIVAL AND MOLTING[1]

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During the last several decades, papers too numerous to list here have been published describing larval stages of decapod crustaceans reared in the laboratory. Through this work techniques for rearing these larvae have become standardized, making a variety of physiological and ecological experiments possible (for example, Costlow, 1961, 1964, 1966, 1967; Costlow and Bookhout, 1968; Roberts, 1971a, 1971b).

Yet relatively little is known about the nutritional requirements of decapod larvae. Lebour (1922) performed a limited number of stomach content analyses on planktonic zoeae and reported that decapod larvae are principally phytophagous. Subsequent laboratory study led her to conclude that, contrary to her first report, many decapod larvae are actually carnivorous and that diatoms and other phytoplankters observed in stomach content analyses were ingested adventitiously (Lebour, 1927; Gurney, 1942).

Hart (1935, 1937) and Sandoz and Rogers (1944) in their early efforts to culture decapod larvae simply concentrated plankton and isolated desirable (or plentiful) components to feed to larvae. Through these and other studies, it has been verified that many decapod larvae require animal material in their diet, although it was suggested that *Callinectes sapidus* may utilize a dinoflagellate (Sandoz and Rogers, 1944).

A standard diet of *Artemia* nauplii has been used by most recent workers for culturing decapod larvae (for example, Costlow and Bookhout, 1960 and numerous more recent studies; Forss and Coffin, 1960; Chamberlain, 1962; Broad, 1957; Regnault, 1969a, 1969b; Provenzano, 1967a, 1967b). This diet has numorous practical advantages for culture works: (1) it is available in large quantities regardless of season, (2) it is a suitable size for many decapod larvae, and (3) it permits complete development to the juvenile or beyond with reasonably consistent mortality rate, intermolt duration, morphogenetic sequence, *etc.*

Nevertheless, *Artemia* is not a "natural diet" and there is no certainty that larvae reared on this diet follow the same morphogenetic sequence as larvae in the plankton. Furthermore, for commercial production operations, *Artemia* represents a high production cost. When planktonic and laboratory-reared specimens have been compared, minor discrepancies in morphological detail have been noted (LeRoux, 1966; Roberts, 1968; McDonald, Pike and Williamson, 1957; Bookhout, 1964; Chamberlain, 1962). This is quite different from the variability in number of instars observed under presumably constant culture conditions among the carid shrimp (Broad, 1957; Hubschman, 1969; LeRoux, 1963; Regnault, 1969a) and *Upogebia* (Webb, 1921; Roberts, unpublished).

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A few authors have investigated the food value of specific unicellular algae and zooplankters for decapod larvae selected on the basis of availability. Algae prolong survival but do not permit complete development (Broad, 1957a; Chamberlain, 1962; Regnault, 1969b), except for certain species of *Pinnotheres* (Atkins, 1955), *Thor floridanus* (Broad, 1957b) and penaeid shrimp (Cook and Murphy, 1969). Sandoz and Rogers (1944) indicated that a dinoflagellate was a major prey species in their food mixture used for rearing *Callinectes sapidus.* No studies of the possible role of algae in the nutrition of anomurans have been conducted.

Copepods and other zooplankters promote complete development. Studies with these organisms have been hampered by technical difficulties in providing various diets in sufficient quantity when needed. Nauplii of *Artemia* have been selected as the standard laboratory diet for decapod larvae to overcome these difficulties, even though it is not a "natural" diet.

This paper describes some preliminary studies of suitable diets for the larvae of the anomuran, *Pagurus longicarpus,* conducted at the Virginia Institute of Marine Science, Gloucester Point, Virginia. Responses of larvae presented with several potential food sources were observed directly. Selected diets which could be provided in quantity over a suitably long period were then tested in culture experiments to determine whether larvae could survive and develop to or beyond the megalopa.

Materials and Methods

Direct observations of feeding by *P. longicarpus* larvae were made using a dissecting microscope. Several zoeae grown in mass culture to various stages of development were placed in a Syracuse watch glass with *ca.* 10 ml filtered sea water containing a number of food organisms and observed periodically for at least one hour. Care was taken not to heat the container with the microscope illuminator. The food organisms supplied were copepod nauplii, copepodites, *Balanus* nauplii, *Polydora* 3–5 setiger larvae, and pelagic stages of several polychaete worms (unidentified). These organisms were all collected from plankton in the seawater system.

In a series of three culture experiments, selected algae and invertebrate larvae were used to feed larvae of *P. longicarpus.* Mortality rate and intermolt duration for larvae fed experimental diets were compared to those for unfed and *Artemia* nauplii fed control groups. The control *Artemia* diet is known to permit complete development of *P. longicarpus* larvae (Roberts, 1970, 1971a, 1971b).

The experimental algal diets were (1) a mixture of microflagellates (*Cyclotella, Dunaliella,* and *Monochrysis*) and (2) the dinoflagellate, *Amphidinium klebsii.* Microflagellates, cultured by the Microbiology Department, were used in Experiment 2. A volume of algal culture was added to filtered sea water to give a final algal concentration of *ca.* 10^4 cells/ml. The dinoflagellate, *Amphidinium,* used in Experiment 3, was grown in mass culture on an enriched sea water medium. During the course of the experiment, the culture was found to be contaminated with *Dunaliella* (20–50%). No estimate of the concentration of dinoflagellate provided each zoea was made, although some cells always remained at the end of each

24 hour period. In neither case were the algal cells washed free of the original culture medium before use.

The animal diets tested were shelled larvae of *Crassostrea virginica* and the post-trochophore stage of *Arenicola marina*. The *Crassostrea* larvae, obtained from oysters collected at Horsehead, Virginia were cultured by the Malacology Department. The larvae used in Experiment 1 ranged from early umbo stage (80–150 μ) to presettlement stage (250–320 μ). Approximately 20–40 larvae were given to each zoea daily. The *Arenicola* post-trochophores used in Experiment 3 were obtained as follows: several gelatinous egg strings were collected daily at low tide from the beach in front of the laboratory, washed in filtered sea water, and placed in sea water in large finger bowls. The post-trochophores emerged from the egg strings and accumulated on the lighted side of the culture dish. These larvae were sticky, adhering to the glass dish and to each other. In addition they carried with them some diatoms (*Nitschia sigmoides*) which were numerous in the egg strings. To remove the gelatin and diatoms, the larvae were washed by vigorous pipetting in filtered sea water. This procedure was not completely successful since many *Arenicola* post-trochophores still adhered to the culture vessels used in the feeding experiments. Each *Pagurus* zoea was given 200–400 *Arenicola* post-trophores daily.

The experiments were carried out in compartmented plastic boxes. The general procedures for obtaining and handling *Pagurus* larvae, both control and experimental groups, were described previously (Roberts, 1970, 1971a). The range of salinity was 18–20‰ (mean: 19‰) in Experiment 1, 20 to 21‰ (mean: 20.5‰) in Experiment 2, and 22 to 23‰ (mean: 22.3‰) in Experiment 3. The cultures were maintained at ambient sea water temperatures on a sea table. The range of temperature was 18 to 22° C (mean: 21° C) in Experiment 1, and 24 to 28° C (mean: 26° C) in Experiments 2 and 3. These conditions were within the acceptable range for this species (Roberts 1971a). The larvae were examined daily and the presence or absence of material in the digestive tract was noted. In all experiments, food was in excess. No efforts were made to quantify the amount ingested.

RESULTS

Pagurus zoeae were observed to feed successfully on copepod nauplii, copepodites, *Balanus* nauplii, *Polydora* 3–5 setiger larvae and pelagic stages of several unidentified polychaete worms. The zoeae handled each of these much the same way as *Artemia* nauplii and *Arenicola* post-trochophores (see below). Further testing of these potential foods was not feasible because of the time required to procure adequate amounts of the experimental foods.

Larvae in the unfed control group always had empty guts. These larvae never molted to Zoea II in any experiment. The survivorship curves for the unfed control group in the three experiments show no major differences. In all experiments there was a period of high survival lasting 3–4 days (slightly less in Experiment 1) followed by high mortality. Only 50% of the population was alive after 3.5–4.2 days. All of this group were dead by day 5–7.

Larvae in the *Artemia*-fed control group always had some material in the gut. Direct observations of feeding revealed that each *Artemia* nauplius is captured by the maxillipeds by chance encounters during normal swimming activity and pushed

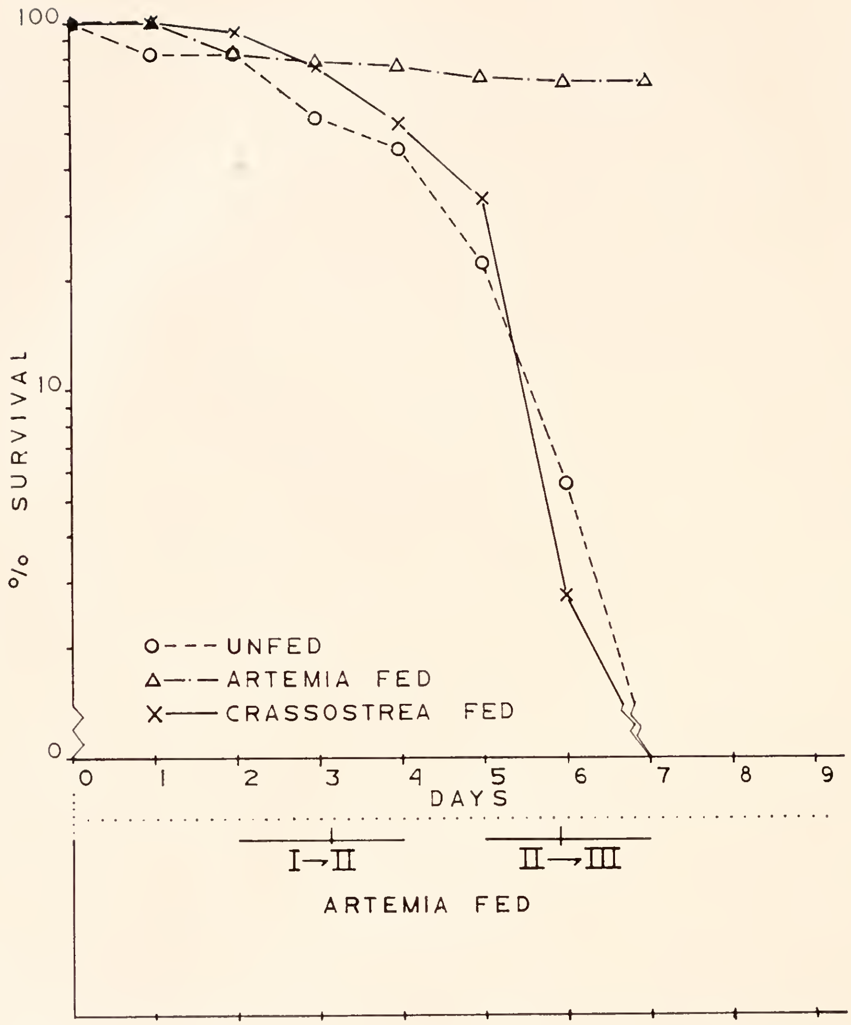

FIGURE 1. Survivorship curves for Experiment 1 comparing *Pagurus* larvae fed oyster larvae, a control group fed *Artemia* nauplii, and an unfed control group. Below the survivorship curve is a bar graph indicating the time during which molting occurred and the mean time to molt.

against the maxillae, maxillules and mandibles. The mandibles "chew" the nauplius, but generally only one or more appendages are removed before the nauplius escapes. Late stage zoeae are more efficient, but still rarely ingest an entire nauplius. Fecal production was undetected during the early stages, pronounced in late Zoea III and Zoea IV. The *Artemia*-fed control group always exhibited a high rate of survival. Experiment 1 was terminated after day 7 and when 53% of the remaining larvae had reached the third instar. In Experiment 2, 31% of the larvae reached the megalopa after a mean of 10.5 days, in Experiment 3, 58.9% after 9.9 days.

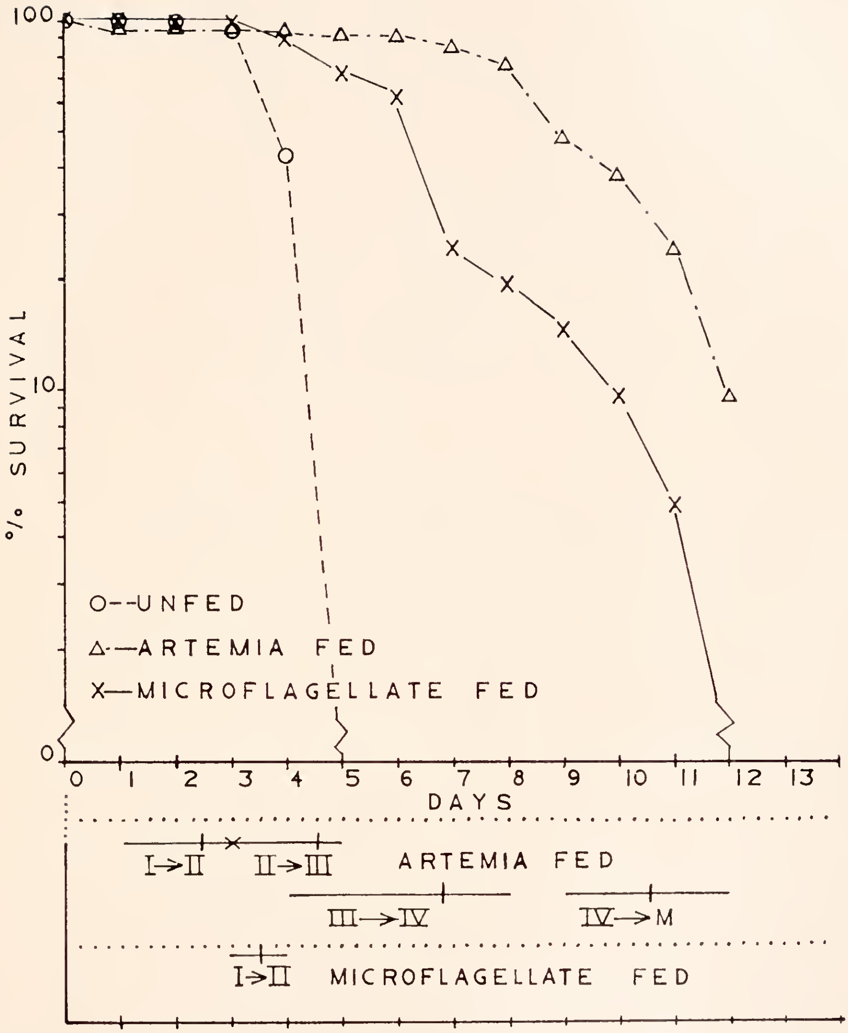

FIGURE 2. Survivorship curves for Experiment 2 comparing *Pagurus* larvae fed microflagellates and control groups. Below the survivorship curves is a bar graph indicating the molt record.

The algal diets were only marginally suitable for larval culture. Both were ingested by zoeae of *P. longicarpus* as indicated by the green color of the gut but details of ingestion could not be observed. The bulk of the ingested material seemed to be defecated. Both algal diets permitted a small number of zoeae to molt to the second instar (9.5% and 11.1%). The *Amphidinium*-fed and unfed control groups had nearly identical survivorship curves (Figure 3) until day 5 when the curve for the *Amphidinium*-fed larvae leveled off following the molt to Zoea II. Too few larvae survived to provide information on the value of *Amphidinium* as a food for Zoea II and older *Pagurus* larvae.

Shelled *Crassostrea* larvae proved unsuitable as food for *Pagurus* larvae. Zoeae which captured an oyster larva with their maxillipeds were unable to break the shell with the maxillae, maxillules and mandibles. Oyster larvae would frequently close their valves on the maxillae thus impeding further feeding activity and to some extent swimming of the larva. The inability to deal with oyster larvae was not an artifact of the method of observation since in culture, oyster-fed zoeae had a survivorship curve nearly identical with that of the unfed control group. The unfed control group and oyster-fed group exhibited 50% mortality on day 3.5 and 4.1 respectively, with 100% mortality on day 7 for both groups, while 68% of the *Artemia*-fed control group survived and molted to Zoea III (Figure 1).

Arenicola post-trochophores were readily handled by *Pagurus* zoeae and, in contrast to *Artemia* nauplii, were usually totally ingested once captured. This diet was the only one, aside from *Artemia* which permitted complete development to the megalopa. The mean post-hatch time and intermolt duration for *Artemia*-fed and *Arenicola*-fed groups are given in Table I. The intermolt durations for the *Artemia*-fed control group increased from 1.57 days for Zoea I to 3.70 days for Zoea IV, while the intermolt duration for *Arenicola*-fed zoeae was uniformly 3.1–3.6 days for each instar. This trend of increasing intermolt duration from Zoea I to IV for *Artemia*-fed larvae, although not as uniformly progressive, was noted previously (Roberts, 1971a). The consistently long intermolt duration for *Arenicola* post-trochophores may reflect inadequate amounts of food in the early zoeal stages. With regard to mortality, the survivorship curve for these two groups is not significantly different until day 10, which corresponds to the molt of the *Artemia*-fed group from Zoea IV to megalopa, at which time they showed a marked decrease in survival rate. This break in the survivorship curve for

TABLE I

Mean post-hatch time and intermolt duration for Artemia-*fed (control) and* Arenicola-*fed (experimental) larvae (from Experiment 4)*

Instar	*Artemia*-fed		*Arenicola*-fed	
	Mean posthatch time	Intermolt duration	Mean posthatch time	Intermolt duration
I		1.57		3.34
	1.57		3.34	
II		2.16		3.10
	3.73		6.44	
III		2.47		3.06
	6.20		9.50	
IV		3.70		3.63
	9.90		13.13	
M				

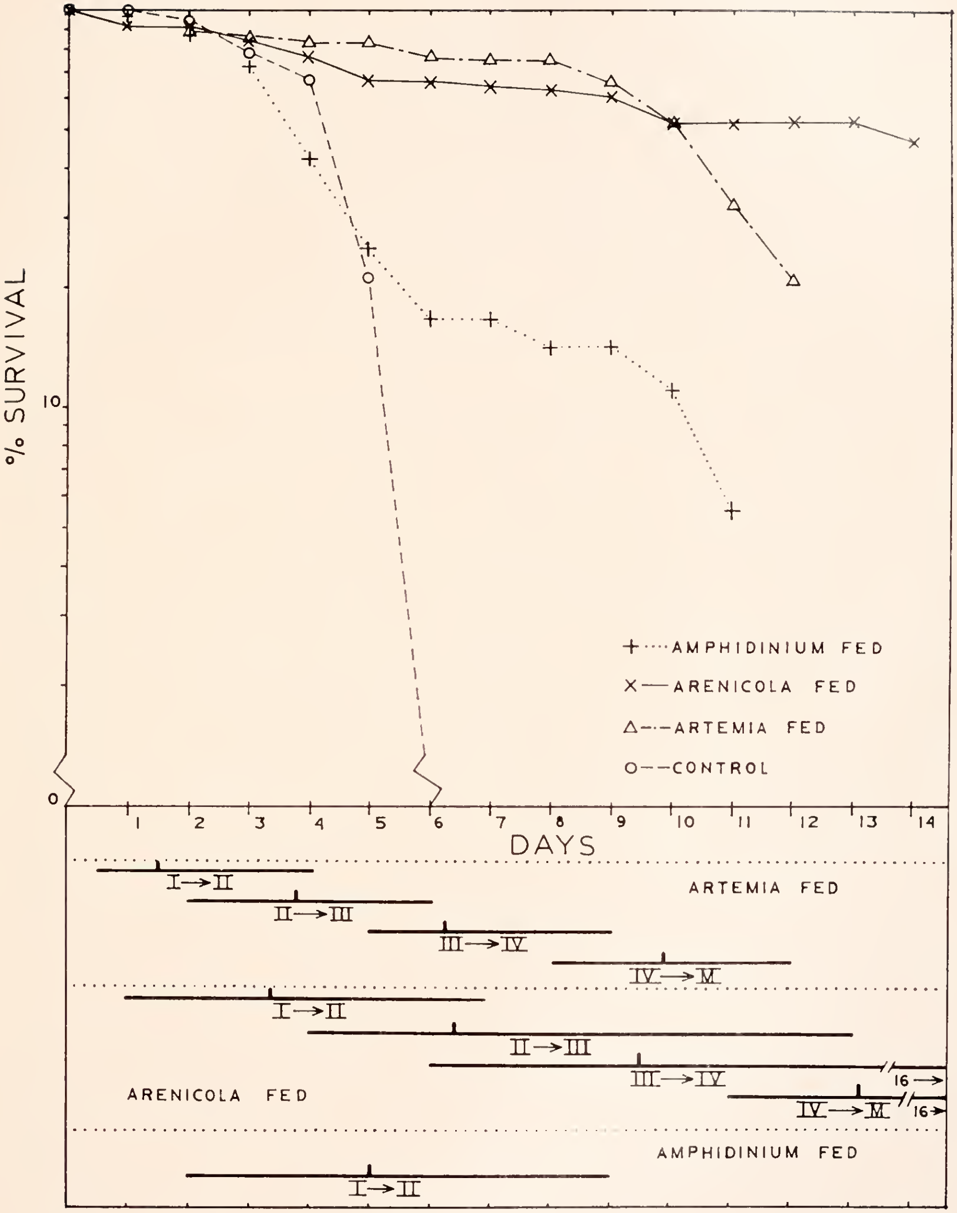

FIGURE 3. Survivorship curves for Experiment 3 comparing *Pagurus* larvae fed *Amphidinium* or *Arenicola* larvae to control groups. Below the survivorship curves is a bar graph indicating the molt record.

Artemia-fed larvae (Figures 2 and 3) was also noted previously (Roberts, 1971a). No similar decline in survival of *Arenicola*-fed larvae was associated with this molt (Figure 3).

DISCUSSION

Unfed larvae of this and other decapod species survive a period slightly greater than the normal intermolt duration at the first instar. Only carid shrimp are reported to exhibit some molting to the second instar when unfed (Regnault, 1969b; Broad, 1957a; Roberts, unpublished). Regnault (1969a) demonstrated an inverse correlation between starvation resistance of *Hippolyte inermis* and temperature. Rice and Provenzano (1966) obtained a similar result for *Dromidia antillensis.* However, Provenzano (1967b) obtained the opposite result for *Trizopagurus magnificus.* Regnault (1969a) also presented evidence that eggs produced early in the year had greater yolk reserves than those produced later. In the study reported here there was no conclusive evidence of a relationship between starvation resistance and temperature or season.

The *Artemia* diet promoted complete development to the megalopa. The survival rate was very high until the Zoea IV-Megalopa molt at which time mortality increased rapidly. The intermolt duration of Zoeae IV was always prolonged, and in Experiment 3, the intermolt was progressively longer for each instar. This phenomenon has been reported previously (Roberts, 1971a). Bookhout and Costlow (1970) have reported that larvae of four brachyuran species fed *Artemia* nauplii hatched from eggs collected in Utah had poorer survival than larvae fed *Artemia* hatched from eggs collected in California. Further, two species (*Rhithropanopeus harrisii* and *Callinectes sapidus*) showed some abnormalities in the megalopal or first crab stages. They also reported the concentration of DDT in *Artemia* nauplii hatched from cysts collected in Utah to be three times higher than in those from cysts collected in California. These observations may explain the discontinuity in the survivorship curve observed in the present study in which the *Artemia* cysts originated from Utah.

The microflagellate and dinoflagellate species tested were not suitable diets for *Pagurus longicarpus* in that they did not promote complete development. There were some benefits of an algal diet in prolonging survival and permitting a few animals to molt once. One might suspect that this relates to the quantity of material provided by an algal diet rather than quality. The fact that the ingested algae are largely defecated whereas animal material is nearly completely assimilated suggests that the deficiency of algal diets is a matter of nutritive value.

Crassostrea larvae (or other molluscan larvae) can be provided in the laboratory in adequate quantities by use of techniques developed by Loosanoff and Davis (1963) and their co-workers. These would therefore seem quite suitable as food for decapod larvae in culture. The inability of *Pagurus* larvae to ingest oyster larvae was unexpected since Lebour (1927) and Hart (1937) had reported using *Ostrea* larvae as food for decapod larvae. These workers may have used only *Ostrea* trochophores rather than shelled larvae. Decapod larvae are probably incapable of ingesting any shelled larvae of molluscs and hence molluscan larvae used as food for crustacean cultures would have to be produced daily.

Arenicola post-trochophores are smaller than *Artemia* nauplii and hence may be handled more readily. They proved equally nutritious for pagurid larvae and did not cause increased mortality after Zoea IV which was observed among *Artemia*-fed larvae. However, *Arenicola* is not especially valuable for culture studies since they are only seasonally available, and are not as readily caught because they tend to adhere to the culture dish. Larvae of polychaete annelids may be very significant in the diet of naturally occurring decapod larvae as they are very abundant during the breeding season of most estuarine decapods.

Pagurus larvae have been observed to ingest several other organisms in the laboratory. Nauplii of *Balanus* were readily ingested as were small copepods and copepod nauplii. No culture experiments were attempted because these organisms were not available in sufficient quantity. Chamberlain (1962) tested the value of copepod nauplii as food for *Rhithropanopeus* zoeae and found them equal in value to *Artemia* nauplii. Copepod nauplii were obtained from the plankton, which is a very tedious and uncertain process.

Techniques are available which permit continuous culture of selected calanoid copepod species in the laboratory though not yet in quantities sufficient to feed crustacean, fish, or other carnivorous larvae (Zillioux, 1969; Zillioux and Lackie, 1970). A copepod diet may be desirable when culturing decapod species which cannot handle food the size of *Artemia* nauplii or species which are especially sensitive to pesticide residues found in *Artemia* or when the experimental design requires a "natural" diet. Naturally occurring decapod larvae must ingest large quantities of copepods, perhaps the single most abundant zooplankter.

Many fish and invertebrates consume detritus when their normal diets are unavailable. In some cases detritus has been found to be a major source of nourishment. Much of this material is decomposing fecal material (Darnell, 1968).

Broad (1957a) used non-living animal material as a source of food in his study of dietary requirements of *Palaemonetes pugio*. He collected zooplankton (presumably including much fecal material), and killed it by immersion in distilled water. This dead animal material was then fed to zoeae. He also tried macerated gonad of *Nassarius*. This diet permitted complete development but was not as good as *Artemia* nauplii though much better than algae or no food. Broad made no attempt to control decomposition of the food. If bacterial growth had been controlled, perhaps his results with non-living material would have been better. Nevertheless, non-living material may be significant in the diet of naturally occurring larvae.

If decapod larvae can develop on a diet of non-living material, then artificial diets may be suitable. Hubschman and Schmitt (1969) reported an attempt to develop a defined diet for larval *Palaemonetes kadiakensis*. This medium permitted development to Form III larvae, whereas unfed larvae only reach Form II in this species. If an artificial diet can be developed, the dietary requirements of decapod larvae can then be studied in detail. However, the necessity of maintaining sterile cultures when using non-living diets precludes the general use of such foods except in large volume cultures where anaerobic conditions are not likely to develop.

Miss Sandra K. Pierson assisted in the collection of the experimental data described here while a NSF Undergraduate Research Participant at the Virginia Institute of Marine Science. Mr. Michael Vaillant prepared the figures. I also wish to thank Mr. Willard A. Van Engel, and Drs. Langley Wood, Vernon C. Goldizen, and Frederick A. Kalber for their helpful discussion and criticism of the manuscript.

SUMMARY

The value of several flagellate algal species, shelled oyster larvae (*Crassostrea virginica*) and annelid larvae (*Arenicola marina* post-trochophores) as food for larvae of *Pagurus longicarpus* was determined. The flagellate algae were ingested by *Pagurus* larvae but did not allow development to the second instar. Shelled oyster larvae were an inadequate diet because *Pagurus* larvae could not break the shell. *Arenicola* post-trochophores allowed complete larval development with survival to the fourth zoeal stage comparable to *Artemia*-fed controls. Survival of *Arenicola*-fed larvae through the megalopal stage was superior to *Artemia*-fed controls. *Pagurus* larvae were shown to be capable of ingesting several microcrustaceans and polychaete larvae, but culture tests of suitability for complete development were not performed.

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SURVEY OF GENETIC DIFFERENTIATION IN A COASTAL ZONE INVERTEBRATE: THE ECTOPROCT *SCHIZOPORELLA ERRATA*

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Wise management of shallow marine and estuarine areas—the coastal zone—will require judgments on anticipated changes in the genetics of marine organisms due to the effects of heated waters from power plants, of chemical pollution from solid waste disposal, *etc.* Changes in gene frequencies in local populations will be caused by natural selection acting through these new environmental influences, and such changes will need to be monitored once baseline data for coastal zone species have been established.

A significant result of both laboratory and natural experiments of the past quarter century has been the documentation of the rapidity with which gene frequencies change in local populations. This is well documented in the extensive studies on *Drosophila* (tabulated in Lewontin, 1974). And if this is the case in the fruit fly, then what about other species which, often for reasons of historical accident, are not as well studied?

Data are presented in this paper in order to establish baseline information on certain genetic characteristics of a prominent coastal zone species. Collections are from 14 localities on Cape Cod taken during the summer and early fall of 1972, and repeated in August-early September, 1973. I have used the most abundant and widely distributed marine, subtidal species of docks and pilings—the ectoproct *Schizoporella errata*. *S. errata* is an orange-red encrusting species that builds a calcium carbonate skeleton as it slowly grows out over the substrate. *S. errata* easily overgrows barnacles, worm tubes, and other sessile animals that happened to settle in its path.

S. errata is a useful organism for coastal zone population genetics because of its natural abundance and its coloniality; this would permit laboratory and field experiments using clones grown from particular genotypes. In the Woods Hole region, this species produces larvae from about July 1 into September. Single colonies have clearly delineated borders. When 2 colonies grow into each other, each stops growth and a sharp line forms between them and this is easily observed in the field. A single colony (or genetic individual) is usually an annual event, but some colonies are able to overwinter and to resume growth the following spring. These colonies form the breeding stock early in the season. Larvae can not feed in the plankton and consequently must settle within a few hours. Mean larval transport per generation is probably less than 1 km, and perhaps much less.

Previous studies cited below have established that *Schizoporella errata* routinely outbreeds; that a local, interbreeding population is likely to be over an area no less than on the order of 10 × 10 m; that no significant selection routinely occurs on exposed *versus* protected sides of pilings correlated with degree of wave action;

TABLE I

Data of ecological interest for localities sampled

Localities	Approximate temperature at warmest season—° C	Sill depth m, low water	Distance to open circulation km	Tidal circulation
(1) Cuttyhunk Channel	20.5	none	open	excellent
(2) Cuttyhunk Harbor	21.5	2.5	0.7	fair–good
(3) Robinsons Hole	20.0	none	open	excellent
(4) Sheep Pen Harbor	22.0	none	open	excellent
(5) Woods Hole	22.5	none	open	excellent
(6) Quissett Harbor	23.5	(2.5)	1.3	fair
(7) Phinneys Harbor (Monument Beach)	23.0	2.0	0.6	fair–good
(8) Green Pond	23.0	2.5	0.8	fair
(9) New Seabury	24.0	1.0	2.0	poor
(10) Lewis Bay (Hyannis)	23.5	3.0	4.2	good
(11) Uncle Roberts Cove	26.5	1.5	4.6	fair
(12) Bass River Inlet	25.0	none	open	good
(13) Stage Harbor	24.0	2.5	1.1	good
(14) Meetinghouse Pond (East Orleans)	27.0	1.5	17.	poor

that genotype and gene frequencies can remain uniform at a given locality for a year; and that gene frequencies at a biallelic leucine amino peptidase locus appears to vary directly as function of environmental temperature measured at the warmest time of the year at 5 localities (Schopf and Gooch, 1971; Gooch and Schopf, 1970, 1971; Schopf, 1973).

MATERIALS AND METHODS

At each of 14 localities on Cape Cod, Massachusetts, and the adjacent islands, I collected by free diving 23 to 83 individual colonies of *Schizoporella errata* in 1972 and again in 1973 from pilings and floating docks. Ecological data (Table I) on temperature, tidal mixing, and distance to the open exchange with the ocean were obtained from observations during collecting, from topographic maps of the U. S. Geological Survey, from coastal charts of the National Oceanographic and Atmospheric Administration, and, most importantly, from discussions with local fishermen and harbor masters.

Slab electrophoresis (Aardvark industries; Lombard, Illinois) using polyacrylamide was usually performed on 24 individuals per electrophoresis box. Procedures for identifying presumptive gene loci are given in earlier publications (Gooch and Schopf, 1970, 1971). Procedures for staining gels were those reported by Hubby and Lewontin (1966) for leucine amino peptidase (LAP, with the minor change that Fast Black K is added at the same time as the substrate), and by Yang (in Selander, Smith, Yang, Johnson and Gentry, 1971) for glutamate oxalate transaminase (GOT). I will document elsewhere the now fairly extensive data on the degree of genetic variability in *Schizoporella errata* (34 loci, 3 definitely polymorphic, 4 provisionally polymorphic; 9–21% of loci polymorphic) (Schopf, 1974).

Three LAP loci have been reported (Gooch and Schopf, 1970), one of which (LAP-2, hereafter referred to as LAP) is of interest in the present paper. LAP is evidently a monomeric enzyme with homozygotes indicated by a single band (slow or fast mobility on the gel) and heterozygotes indicated by a double band (both the slow and the fast bands). Two GOT loci consistently stain, and of these (GOT-2, hereafter called GOT) is polymorphic and of interest here. GOT appears to be a dimeric enzyme with no activity under normal staining conditions for the slow homozygote. Thus one homozygote (fast) is indicated by a single dark band, and the other homozygote by the absence of a band. Heterozygotes are inferred from a double band which is presumably composed of a lower band (due to one subunit from the fast enzyme) and a middle hybrid band (inferred to be the other subunit from the fast enzyme). The usually expected third band of the heterozygote attributable to a subunit of the slow enzyme gives no activity under experimental conditions. All local populations scored in this manner are within the expectations of the Hardy-Weinberg binomial expansion for a 2 allele model (Schopf, 1974).

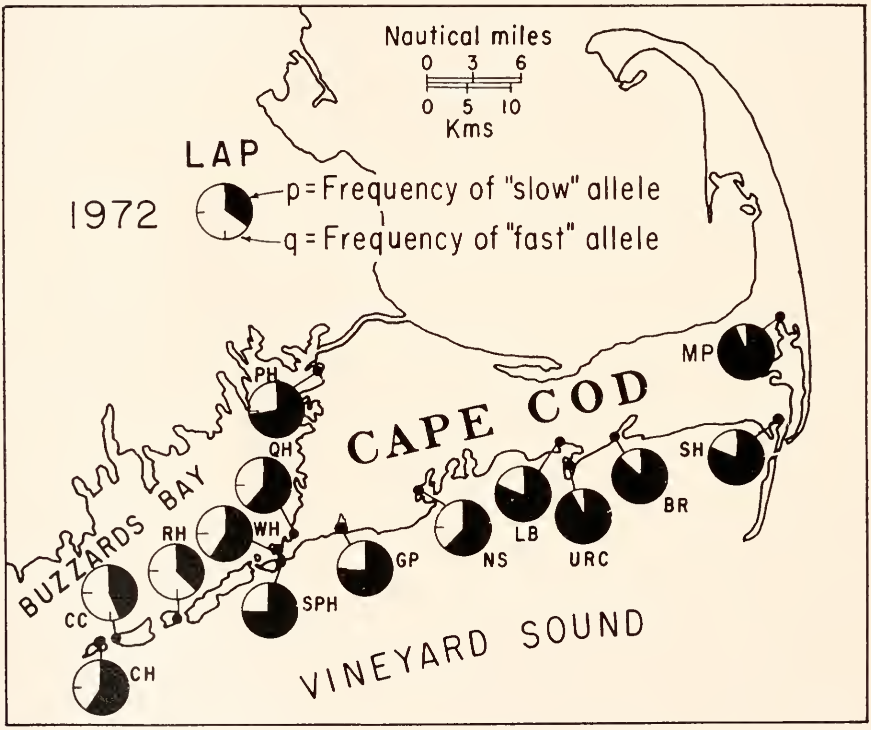

FIGURE 1. Map of allele frequencies (1972) at the polymorphic leucine amino peptidase (LAP) locus. "Fast" and "slow" refer to mobilities of the enzymes on polyacrylamide gels. Note the generally higher frequency of the "fast" allele to the southwest. WH is an average of WH-1 and WH-2. Localities are listed in Table II.

RESULTS

Gene and genotype frequencies for LAP and GOT are reported (Tables II and III), and spatial distributions are plotted (Figs. 1–4). For both 1972 and 1973, a progressive change in allele frequencies for both enzyme loci exists throughout the region studied. Allele frequencies at the LAP and GOT loci also vary in a coordinated manner, and both the absolute change and the rate of change are very similar (Fig. 5).

Water temperature at the warmest time of the year (July through early September) is a good predictor of allele frequencies (Fig. 5). For LAP, R^2, the proportion of total variation about the mean allele frequency explained by regression with temperature, is 64.3% (Draper and Smith, 1966, pages 7–26). For the GOT locus, 68.3% of the variance in allele frequency is explained by water temperature. For comparison, the correlation with distance for the same localities measured away from Cuttyhunk Channel in the southwest explains 24.8% of the variance in allele frequency in LAP, and 40.2% in GOT.

A purely geogrphic interpretation of changes in allele frequencies does not account for observations at adjacent localities that have the same parent body of water, and differ only in their degree of water circulation and hence temperature. In 3 cases, as the temperature hypothesis predicts, the locality with the more restricted circulation shows a shift in allele frequencies toward a higher representation of "warm-water" alleles. These are (1) Cuttyhunk Channel compared with

TABLE II

Genotype and gene frequency for two loci (1972): leucine amino peptidase (LAP) and glutamate oxalate transaminase (GOT). Abbreviations are: n = number of individuals; if two numbers (e.g. *24/25), n for LAP, then n for GOT; slow = slow mobility homozygote; hetero. = heterozygote; fast = fast mobility homozygote; p = frequency of slow allele; and q = frequency of fast allele*

Locality		n	Leucine amino peptidase					Glutamate oxalate transaminase				
			slow	hetero	fast	p	q	slow	hetero	fast	p	q
CC	Cuttyhunk Channel	27	5	14	8	0.44	0.56	2	6	19	0.19	0.81
CH	Cuttyhunk Harbor	41	17	15	9	0.60	0.40	9	17	14.5*	0.43	0.57
RH	Robinsons Hole	24/25	2	14	8	0.37	0.63	1	7	17	0.18	0.82
SPH	Sheep Pen Harbor	47/23	27	18	2	0.77	0.23	0	4	19	0.09	0.91
WH-1	MBL VERRILL Dock	59/24	19	33	7	0.60	0.40	4	8	12	0.33	0.67
WH-2	MBL Intake Dock	83/23	34	37	12	0.63	0.37	3	8	12	0.30	0.70
QH	Quissett Harbor	24	10	9	5	0.60	0.40	3	14	7	0.42	0.58
PH	Phinneys Harbor	24	11	12	1	0.71	0.29	2	8	14	0.25	0.75
GP	Green Pond	24	14	8	2	0.75	0.25	9	9	6	0.56	0.44
NS	New Seabury	24	9	12	3	0.63	0.37	6	11	7	0.48	0.52
LB	Lewis Bay	24	16	7	1	0.81	0.19	3	13	8	0.40	0.60
URC	Uncle Roberts Cove	24	20	4	0	0.92	0.08	11	8	5	0.63	0.37
BR	Bass River	23	17	6	0	0.87	0.13	7	12	4	0.56	0.44
SH	Stage Harbor	24	14	10	0	0.79	0.21	6	10	8	0.46	0.54
MP	Meetinghouse Pond	24	20	3	1	0.90	0.10	9	11	4	0.60	0.40

* includes a "fast allele which occurred in heterozygous state with another (very rare) allele.

Cuttyhunk Harbor (1.5 km distant), (2) Lewis Bay (Hyannis) compared with Uncle Roberts' Cove (2.9 km removed from the open channel), and (3) Stage Harbor (Chatham) compared with its counterpart Meetinghouse Pond (East Orleans), about 22 km "upstream" at the end of a long marine passageway.

Allele frequencies appear to be stable for as long as 5 successive years (Table IV). Data from one year to the next are independent in the sense that at any given locality recruitment by migration and local breeding could form each new population. In fact, some large colonies overwinter and probably would be sampled in successive years. Inclusion of data from overwintering colonies has the effect of damping variations in allele frequencies due to yearly settlement. However this in no way alters the fact that data on allele frequencies represent the living local population for the year sampled.

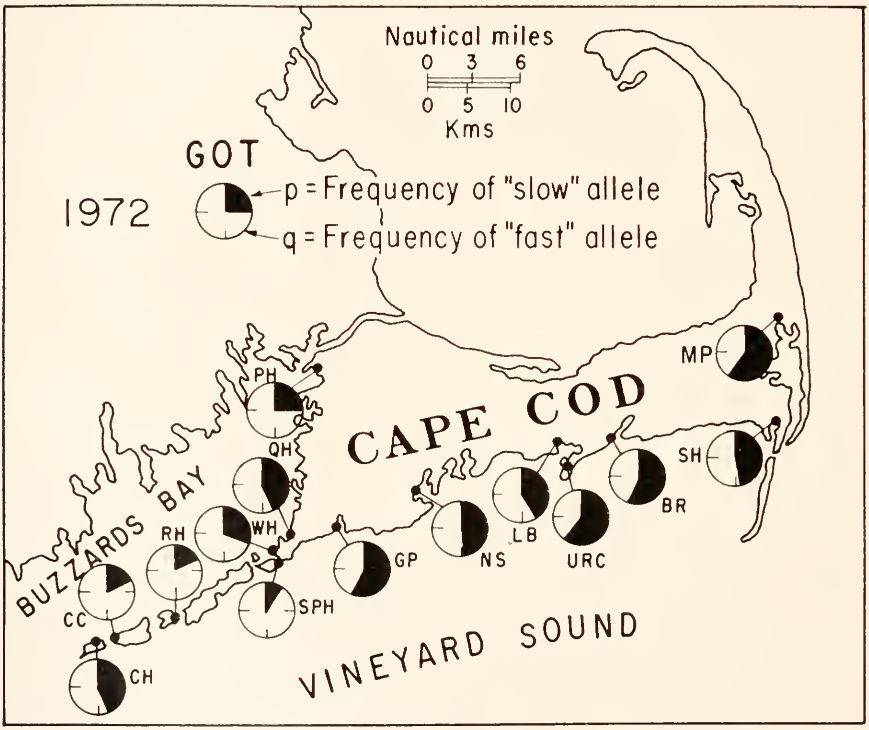

FIGURE 2. Map of allele frequencies (1972) at the polymorphic glutamate oxalate transaminase (GOT) locus. "Fast" and "slow" refer to mobilities of the enzymes on polyacrylamide gels. Note the generally higher frequency of the "fast" allele to the southwest. WH is an average of WH-1 and WH-2. Localities are listed in Table II.

Previous studies suggested the possibility of a significant change in allele frequencies with water depth in LAP (Gooch and Schopf, 1971), and this was further investigated. In a test of homogeneity (Dixon and Massey, 1969, page 241) for LAP at the MBL Water Intake Dock, there is a significant difference comparing samples at 0–2 m ($p = 0.83$) *vs.* 6–7 m ($p = 0.60$), ($\chi^2_{(1)} = 6.2$, $P > 0.975$).

However, at the MBL *Verrill* Dock, a significant difference clearly does not exist comparing samples 0–2 m ($p = 0.62$) *vs.* 3–7 m ($p = 0.67$). For GOT, neither dock shows a difference with depth at the 95% confidence level ($\chi^2_{(1)} = 1.81$, $P > 0.75, < 0.90$; $\chi^2_{(1)} = 2.00$, $P > 0.75, < 0.90$).

DISCUSSION

The observed pattern of gene frequencies (Figs. 1–4) conceivably could be due to (1) historical relict, (2) local continuing selection at each generation, or (3) a combination of these. Perhaps local, total selection at each generation comes closer to accounting for patterns in gene frequencies because of the great ease with which organisms are known to respond to selection pressures (Lewontin, 1974). And the geographic settings in which "warm-water" alleles increase in frequency in more restricted areas have probably existed for only 10 to 100 years (approximately 10 to 100 generations). Presumably the warmer the water becomes over the season, the more favored are the individuals with a compliment of genes that includes the fast, "warm-water" alleles.

TABLE III

Genotype and gene frequency for two loci (1973): leucine amino peptidase (LAP) and glutamate oxalate transaminase (GOT). Abbreviations are; n = numbr of individuals; slow = slow mobility homozygote; hetero. = heterozygote; fast = fast mobility homozygote; p = frequency of slow allele; and q = frequency of fast allele

Locality		n	Leucine amino peptidase					Glutamate oxalate transaminase				
			slow	hetero	fast	p	q	slow	hetero	fast	p	q
CC	Cuttyhunk Channel	24	8	5	11	0.44	0.56	2	7	15	0.23	0.77
CH	Cuttyhunk Harbor	24	10	14	0	0.71	0.29	5	8	11	0.37	0.63
RH	Robinsons Hole	24	4	6	14	0.29	0.71	2	6	16	0.21	0.79
SPH	Sheep Pen Harbor	24	14	9	1	0.77	0.23	1	3	20	0.10	0.90
WH-1	MBL VERRILL Dock											
	0.5–2.0 m	24	10	10	4	0.62	0.38	2	3	19	0.15	0.85
	3.0–7.0 m	23	9	13	1	0.67	0.33	3	6	14	0.26	0.74
WH-2	MBL Intake Dock											
	0.5–2.0 m	24	16	8	0	0.83	0.17	4	11	9	0.40	0.60
	6.0–7.0 m	24	9	11	4	0.60	0.40	2	6	16	0.21	0.79
QH	Quissett Harbor	24	11	8	5	0.62	0.38	5	8	11	0.38	0.62
PH	Phinneys Harbor	24	13	10	1	0.75	0.25	5	7	12	0.35	0.65
GP	Green Pond	24	14	7	3	0.73	0.27	7	10	7	0.50	0.50
NS	New Seabury	24	11	6	7	0.58	0.42	7	11	6	0.52	0.48
LB	Lewis Bay	24	14	10	0	0.79	0.21	4	5	15	0.27	0.73
URC	Uncle Roberts Cove	24	18	6	0	0.88	0.12	14	8	2	0.75	0.25
BR	Bass River	24	17	7	0	0.85	0.15	10	10	4	0.63	0.37
SH	Stage Harbor	24	11	11	2	0.69	0.31	5	11	8	0.44	0.56
MP	Meetinghouse Pond	24	19	5	0	0.90	0.10	11	7	6	0.60	0.40

For the 14 localities mentioned above, selection is thought to operate during the warmest season because all localities undergo similar low temperatures during the coldest time of the year. In addition, as the regional temperature gradient is reversed south of Cape Cod, the "warm-water" allele of LAP is again in high frequency in Delaware, and is fixed at Beaufort, North Carolina (Schopf and Gooch, 1971; Gooch and Schopf, 1971).

I have also obtained LAP and GOT data in 1972 and 1973 for Provincetown, Massachusetts, which is on the north side of Cape Cod and which receives its circulation from the cold water of Cape Cod Bay (summer temperature about 12° C). Three and possibly 4 LAP alleles occur in the Provincetown population, with the allele of highest frequency being of identical mobility as the "cold-water" allele found along southern Cape Cod. However, the water in the Provincetown Harbor where the material was collected is quite warm in the late summer (about 22° C). And in addition the mobility of the allele fixed at the GOT locus is the same as that of the "cold-water" one, both from the transect along the southern shore of the Cape, and, surprisingly, at Beaufort, North Carolina. I have run material from Beaufort, Woods Hole, and Provincetown on the same gel to establish this fact. An additional complicating factor is that the GOT "warm-water" allele is represented in homozygous state on southern Cape Cod by the null activity (as

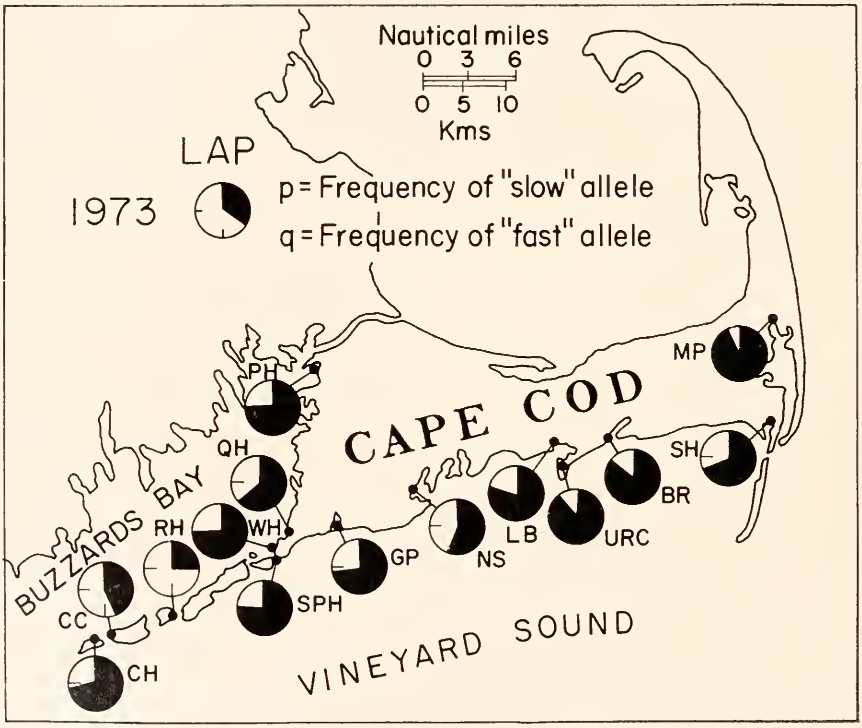

FIGURE 3. Map of allele frequencies (1973) at the polymorphic leucine amino peptidase (LAP) locus. "Fast" and "slow" refer to mobilities of the enzymes on polyacrylamide gels. Note the generally higher frequency of the "fast" allele to the southwest. WH is an average of the WH-1 and WH-2 shallow localities. Localities are listed in Table III.

discussed in Materials and Methods). Biochemical data on these enzyme products would perhaps resolve this issue. From an ecologic point of view, localities in different faunal provinces, as at Provincetown, are probably best compared with places of similar currents, food sources, and water history since the mechanism whereby selection would be acting to cause the observed gene frequencies has not been demonstrated biochemically.

Leucine amino peptidase cleaves N-terminal residues from proteins. Glutamate oxalate transaminase catalyses the reaction of α-ketoglutaric acid + aspartic acid $\rightleftharpoons$ glutamic acid + oxaloacetic acid, and acts to bring aspartic acid into the tricarboxylic acid cycle (Lehninger, 1970). No kinetic data exist on the activity of these enzymes produced by different alleles in ectoprocts. Such data could be used to test the ecologic interpretations presented in this paper.

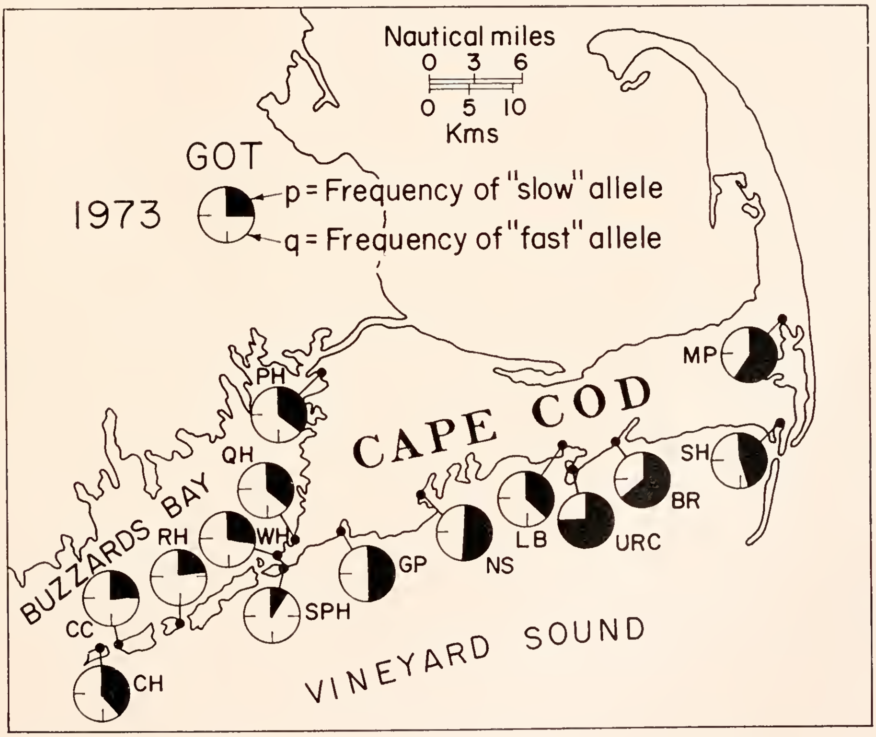

FIGURE 4. Map of allele frequencies (1973) at the polymorphic glutamate oxalate transaminase (GOT) locus. "Fast" and "slow" refer to mobilities of the enzymes on polyacrylamide gels. Note the generally higher frequency of the "fast" allele to the southwest. WH combines WH-1 and WH-2 shallow localities. Localities are listed in Table III.

Since LAP and GOT values at every specific locality are not precisely correlated with each other (see Fig. 5), the differences may be due to selection being somewhat different in the two cases, or to the several types of possible background "noise". Perhaps the species composition of the phytoplankton food of *Schizoporella errata,* which in general varies with water temperature, and yet may differ from

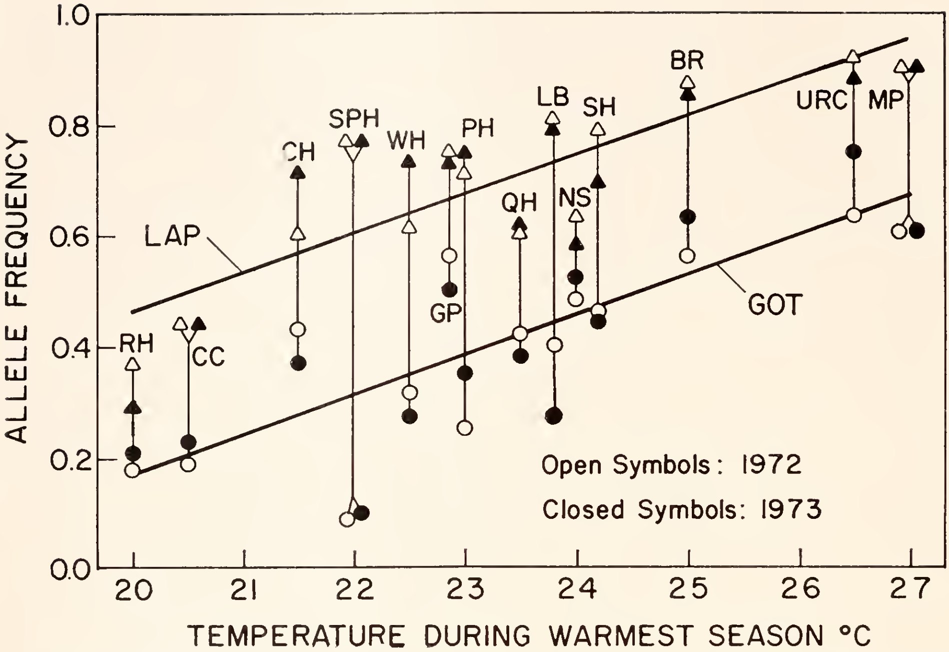

FIGURE 5. Chart of allele frequencies *versus* temperature during warmest season in °C. For each year, points for the two loci (LAP = leucine amino peptidase; GOT = glutamate oxalate transaminase) are from the same organisms collected at the same time, and must necessarily have the same temperature history. If y = allele frequency, and x = temperature, for LAP, $y = -.93 + 0.069x$; for GOT, $y = -1.25 + 0.071x$. Note that the slope of the lines are nearly identical.

place to place, would be especially important by providing differing organic compounds on which the enzymes in question would be acting.

Allele frequencies from successive years are rather close to each other (Tables I–III; Fig. 5). This supports the view that local populations are in some sort of local equilibrium with local ecologic conditions. Fitness surfaces for 1972 and

TABLE IV

Allele frequencies for localities with data for 3 to 5 years. Abbreviations are: n = number of individuals; p = frequency of slow mobility allele; and q = frequency of fast mobility allele

Locality	1969			1970			1971			1972			1973		
	n	p	q	n	p	q	n	p	q	n	p	q	n	p	q
Cuttyhunk Channel				30	0.35	0.65	31	0.39	0.61	27	0.44	0.56	24	0.44	0.56
Robinsons Hole				36	0.31	0.69				24	0.37	0.63	24	0.29	0.71
Sheep Pen Harbor				29	0.62	0.38				47	0.77	0.23	24	0.77	0.23
Woods Hole (VERRILL Dock)	50	0.72	0.28	45	0.61	0.39	58	0.58	0.42	59	0.60	0.40	24	0.62	0.38
Green Pond	43	0.76	0.24	47	0.76	0.24				24	0.75	0.25	24	0.73	0.27
Quissett Harbor							48	0.62	0.38	24	0.60	0.40	24	0.62	0.38
Phinneys Harbor							48	0.80	0.20	24	0.71	0.29	24	0.75	0.25

1973 data have been prepared for warm water, intermediate temperature and cool water localities (Schopf, 1974). These show that there is a change in fitness values for a given genotype in different environments. The importance of selection of different genotypes in different environments was analyzed by Smith (1970) who showed that "a stable polymorphism in a varied environment does not require that the heterozygote is the fittest genotype in any environment, and is in fact possible if one allele is dominant in all environments."

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SUMMARY

Gene and genotype frequencies are reported for two polymorphic loci which make the enzymes leucine amino peptidase and glutamate oxalate transaminase for a ubiquitous coastal zone species, the marine ectoproct *Schizoporella errata* (*S. unicornis* of most literature). Allele frequencies were determined from 14 localities in the Cape Cod region for 1972 and repeated in 1973. Gene frequencies for both loci change in a regular manner that is correlated with local water temperature during the warmest season. Allele frequencies have remained fairly uniform in local populations for as long as 5 successive years.

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MUSCLE ATTACHMENTS IN HORSESHOE CRAB WALKING LEGS

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The attachment of muscle to skeletal elements in arthropods has been the subject of electron microscopical studies in insects (Auber, 1963; Shafiq, 1963; Lai-Fook, 1967; Toselli and Pepe, 1968a, 1968b; Caveney, 1969), arachnids (Smith, Jälfors and Russell, 1969; Kuo, McCully and Haggis, 1971; Mazurkiewicz and Bertke, 1972) and crustaceans (Bouligand, 1962; Talbot, Clark and Lawrence, 1972; Koulish, 1973). The results of these studies reveal that the attachment region is similar in structure throughout the arthropods that have been examined. The attachment may be made directly to the exoskeleton, but in most cases is achieved through a highly specialized epidermal cell, usually called a tendinal cell, interposed between a muscle fiber and the cuticle. The most conspicuous feature of the tendinal cells is their tremendous concentration of oriented microtubules which extend the length of the cell and join desmosomal and hemidesmosomal junctions formed between muscle and tendinal cell and tendinal cell and apodeme respectively.

At the present time, no studies of muscle attachments utilizing electron microscopical techniques have been reported for a fourth major class of arthropods, the Merostomata. Such a study of members of this group which includes the oldest extant arthropods, the horseshoe crabs, would be particularly interesting in view of their long evolutionary history. This report is concerned with the ultrastructural details of the attachment of propodite flexor muscle fibers to the exoskeleton in *Limulus polyphemus* (L.). The results show that for the most part, these muscle attachments in horseshoe crabs follow the pattern described for other members of the Arthropoda.

MATERIALS AND METHODS

Adult female horseshoe crabs, *Limulus polyphemus* (L.), having a carapace width of about 21 cm, were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. The animals were prepared for electron microscopy in March during their intermolt stage. The second, third and fourth pairs of walking legs were used. A leg was removed at the coxopodite, secured to a glass petri dish with dental wax and submerged in artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio) at room temperature. The exoskeleton overlying the propodite flexor muscle heads was removed to expose the regions of muscle attachment at both the origin and insertion ends. A detailed description of the gross morphology and ultrastructure of the propodite flexor muscle is given elsewhere (Fourtner and Sherman, 1972).

After the muscle was exposed, it was fixed *in situ* for two hours in a solution containing 4% glutaraldehyde, 0.1 M sodium cacodylate buffer (ph 7.3), 2% sodium chloride and 0.2% calcium chloride. It then was washed in a solution containing only the above salts and fixed for one hour in 1% osmium tetroxide buffered with 0.1 M sodium cacodylate (pH 7.3). The muscle was again placed in the salts solution and a number of pieces of muscle tissue were removed from different regions of both the origin and insertion ends of the muscle. This was accomplished with a piece of razor blade held by a blade breaker and holder (Model IR-471, Irex Surgical Instruments, Toronto, Canada). The tissues were prestained for two hours in a saturated solution of uranyl acetate in 50% ethanol, dehydrated in a graded ethanol series, cleared in propylene oxide and embedded in an epon-araldite mixture. Thin sections were stained with ethanolic uranyl acetate and lead citrate (Reynolds, 1963) and examined with a transmission electron microscope.

RESULTS

Two modes of muscle fiber attachment occur in the propodite flexor muscle of *L. polyphemus.* At the insertion end, the attachment involves three elements: muscle fibers, tendinal cells and apodemes. At the origin, muscle fibers are attached directly to the exoskeleton by collagen fibrils.

Characteristics of epidermal cells

Sections through apodemes reveal basically two types of epidermal cell. One type, presumably fibroblasts, is embedded in the matrix of the apodeme and probably is responsible for secreting the cuticle (Fig. 1). This cell type is irregular in shape and its nucleus occupies most of the cell volume. The fibroblasts are about 8 to 10 μ at their greatest width. The cytoplasm typically shows a relative scarcity of subcellular structures. In contrast to the second type of epidermal cell, these cells do not form specialized junctions with apodemes or muscle fibers and they do not contain microtubules.

The second type of epidermal cell corresponds to the tendinal cells described previously for other arthropods. These cells are more abundant, contain tremendous numbers of oriented microtubules and form specialized junctions with muscle fibers and apodemes (Fig. 2). They are irregular in shape and interdigitate profusely with muscle and apodemal elements. Besides microtubules and the nucleus, the tendinal cells primarily contain moderate amounts of mitochondria, free ribosomes and single-stranded rough endoplasmic reticulum. Small clear vesicles also are present in most cells. Several tendinal cells often occur in close association with one another (Fig. 3).

The microtubules in the tendinal cells are about 210 Å in diameter. They are not arranged into a particular pattern in the cytoplasm, but usually are within 50 to 150 Å from one another. The microtubules may reach a concentration as high as $910/\mu^2$ in certain cells. They extend the entire length of the tendinal cell which in some cases involves a distance of 20 μ.

In cases where two or more epidermal cells occur together, they are linked by both septate and gap junctions (Figs. 4 and 5). The septate junctions consist of an intercellular gap of about 100 Å and transverse septa spaced about 100 Å apart (Fig. 5). These junctions run uninterrupted for distances of several microns along the borders of neighboring cells. The gap junctions are characterized by a separation of about 30 Å between the apposing membranes at the junctional re-

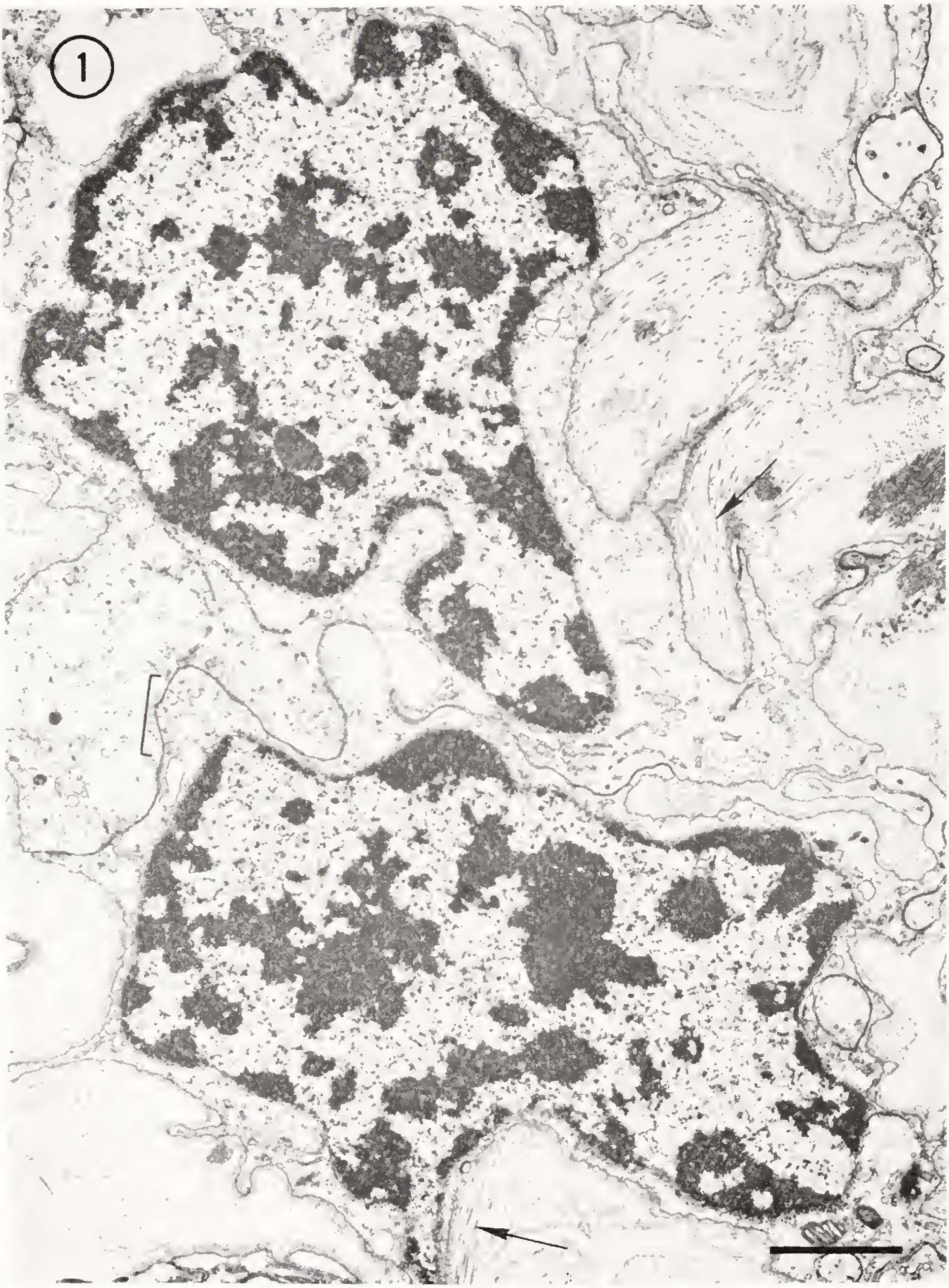


FIGURE 1. Two epidermal cells, presumed to be fibroblasts, are embedded in cuticle. This type of cell lacks appreciable subcellular detail, and the nucleus occupies the bulk of the cell volume. Note the collagen fibrils (arrow) in the cuticle. The area designated by the bracket contains a gap junction which is shown at higher magnification in Figure 4. Calibration line represents 1.3 μ.

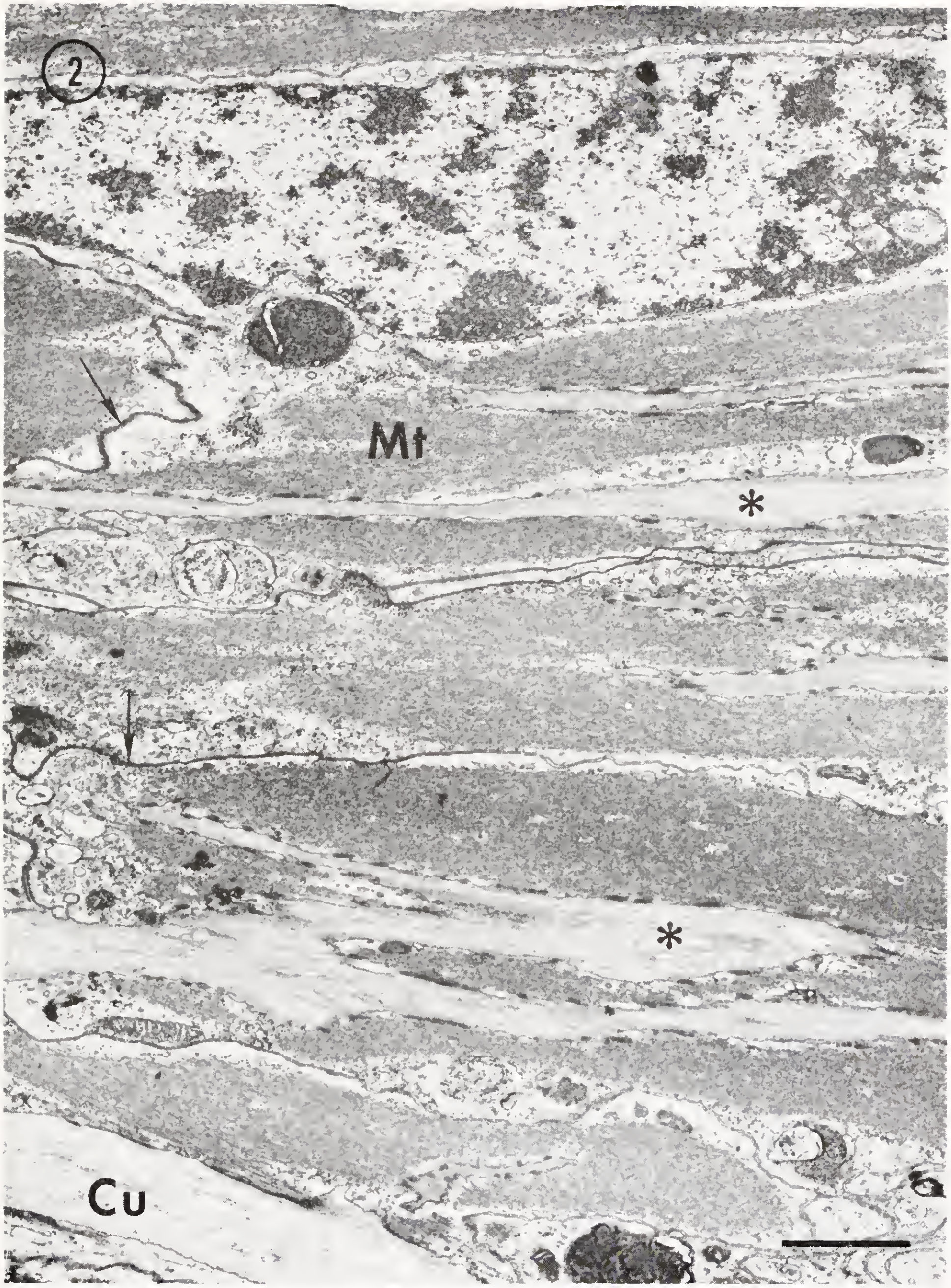
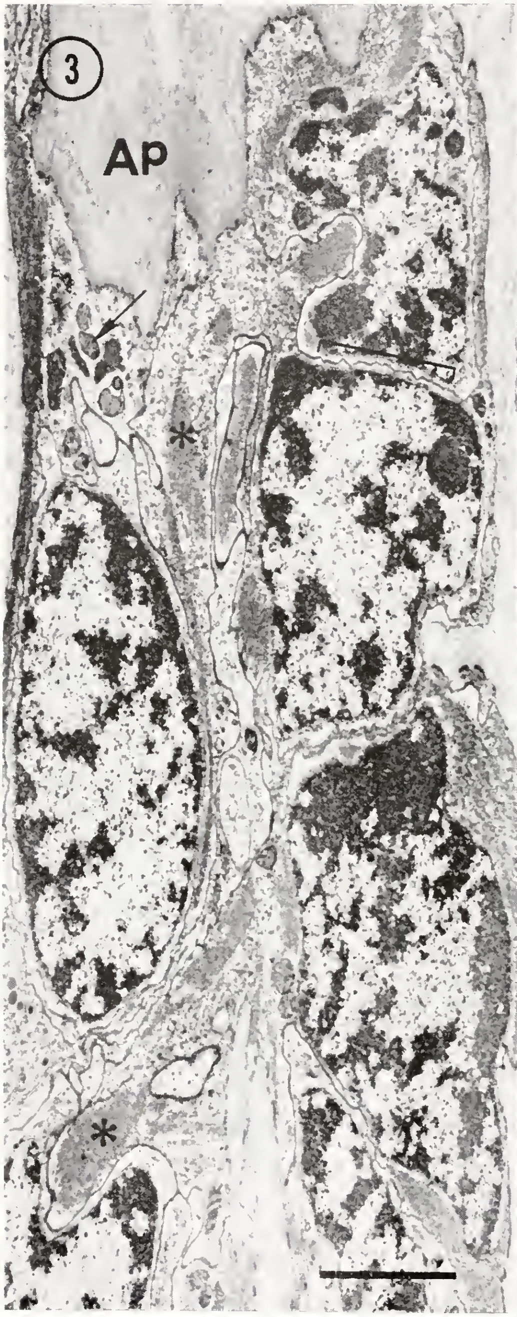
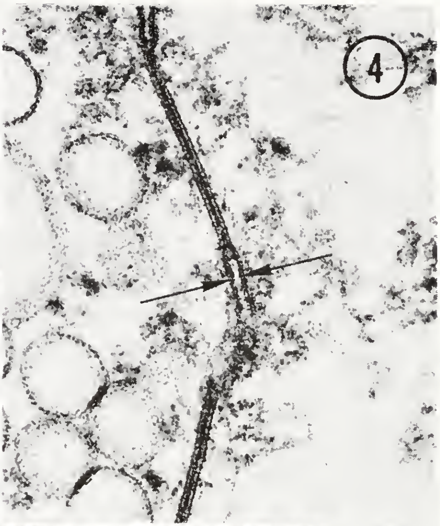


FIGURE 2. Parts of a number of tendinal cells attached to apodeme (asterisks) through hemidesmosomal junctions. The nucleus of one of the tendinal cells is shown as is the close apposition of the membranes of adjacent cells (arrow). Note the tremendous number of microtubules (Mt) oriented longitudinally in the elongated tendinal cells; dense material lines, in a discontinuous manner, the inner surface of the tendinal cell membrane at the regions of attachment. Collagen fibrils are present in the surface cuticle (Cu) but not in the apodemal extensions (asterisks) which have an amorphous appearance. Calibration line represents 1.0 μ.





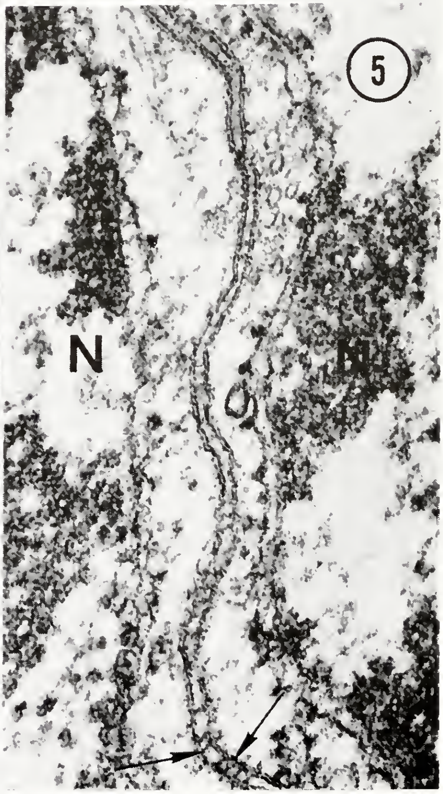


FIGURE 3. Parts of at least six different tendinal cells are shown in close association with one another near an apodeme (Ap). Nuclei, small mitochondria (arrow), very small vesicles and groups of microtubules (asterisks) occur in the cytoplasm. Note the close apposition of the

gions (Fig. 4). An analysis of the three-dimensional structure of these intercellular junctions was not undertaken.

Muscle-tendinal cell junction

The junction between muscle fibers and tendinal cells possesses the structural features of a desmosome and marks the extensive course of the cellular interdigitations (Figs. 6 and 7). Electron-dense material occurs along the inner surface of each apposing membrane at the junction. Thin myofilaments of muscle fibers and microtubles of tendinal cells extend into their respective layers of dense material (Fig. 6). The angle between the thin myofilaments or microtubules and the dense material varies between 15 and 90°. No thick myofilaments are present at the attachment site. Moderately-dense, amorphous basal lamina-like material occupies the space between the cell membranes at the desmosome.

The desmosomes are not continuous across a given muscle fiber. Generally, they are oriented transversely to the longitudinal axis of the muscle fiber, but their course is quite tortuous. The extracellular gap at the desmosome varies in width from about 400 to 1,000 Å. The layer of dense material along the inner surface of the membranes at the desmosome is absent from the neighboring non-junctional areas. Here, membrane invaginations of muscle fibers form tubular elements of the transverse tubular system (Fig. 6).

Tendinal cell-apodeme junction

The junction between the apical end of the tendinal cells and the apodeme is characterized by a considerable degree of invagination of the tendinal cells by "tongue-like" extensions of the apodeme (Figs. 2 and 7). The actual attachment occurs through hemidesmosomes. The microtubules in the tendinal cells connect to the electron-dense material which lines in a discontinuous fashion the inner surface of the tendinal cell membrane at the hemidesmosome. The apodemal indentations into the tendinal cells consist of moderately dense, amorphous material (Figs. 2 and 7). In contrast to the surface cuticle, the apodemal indentations do not contain collagen fibrils.

Direct myo-cuticular attachments

Tendinal cells were not observed at muscle fiber origins. Instead, muscle fibers appear to be connected directly to the exoskeleton by collagen fibrils (Fig. 8). The junction between the collagen fibrils and muscle fibers occurs at the level of

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membranes of adjacent cells. The area designated by the bracket contains a septate junction and is shown at higher magnification in Figure 5. Calibration line represents 1.7 μ.

FIGURE 4. The area designated by the bracket in Figure 1 is shown at higher magnification to show the gap junctions that occur between adjacent epidermal cells. The arrows denote apposing cell membranes. Calibration line in Figure 3 represents 0.17 μ in Figure 4.

FIGURE 5. The area designated by the bracket in Figure 3 is shown at higher magnification to show the transverse septa that occur between certain areas of the membranes (arrows) of adjacent epidermal cells. The nuclei (N) of the two epidermal cells are evident. Unfortunately, no well-preserved septate junctions were located in the present study. Calibration line in Figure 3 represents 0.16 μ in Figure 5.

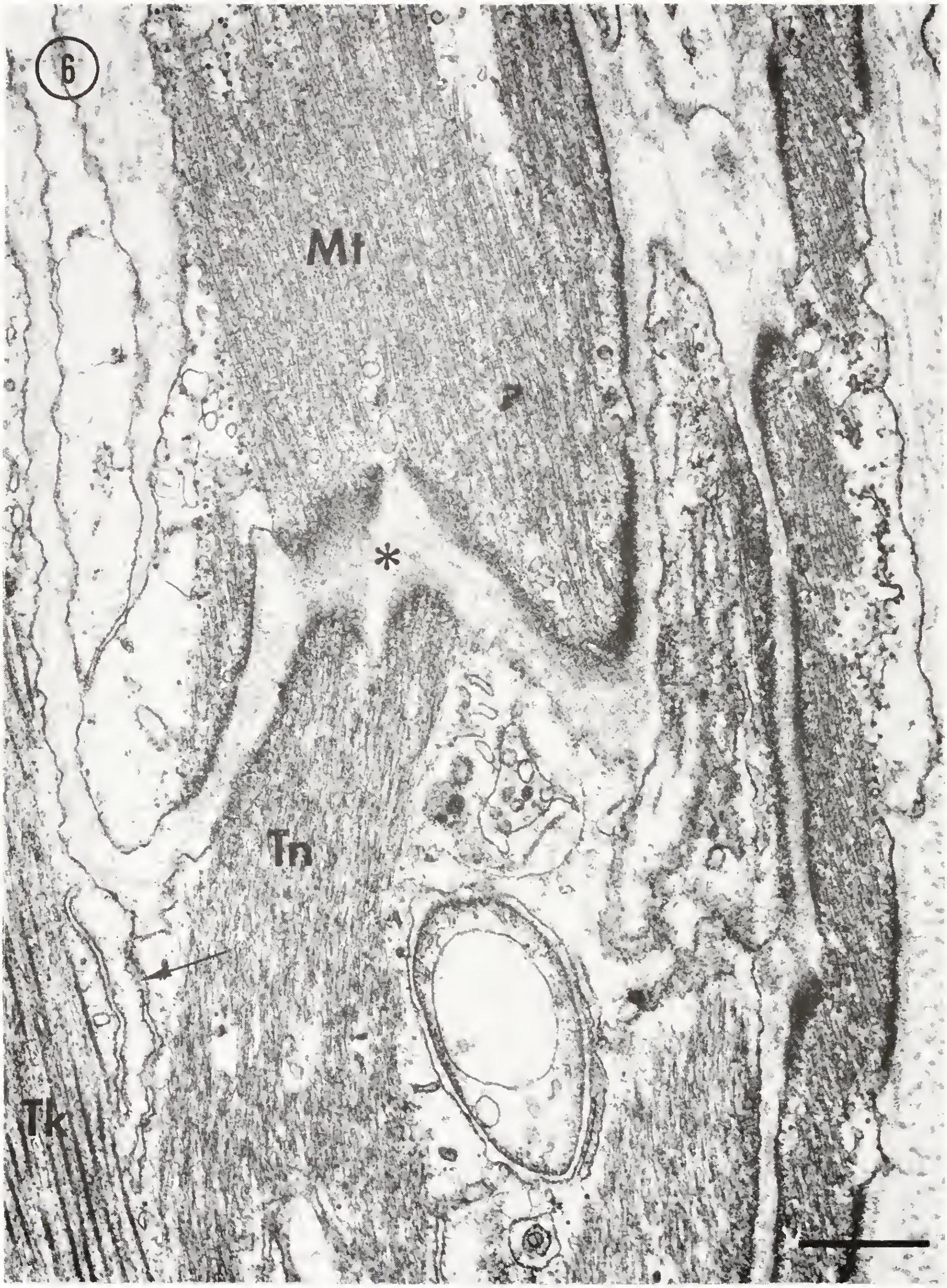

Figure 6. Muscle-tendinal cell desmosomal junction (asterisk) is shown. Microtubules (Mt) of the tendinal cell and thin myofilaments (Tn) of the muscle fiber extend into dense material lining the inner surface of the membranes at the junction. Thick myofilaments (Tk) are not directly involved in the attachment. Note the wide gap between the membranes at the junction. Plasma membrane invaginations (arrow) of the non-junctional region of the muscle fiber form part of the transverse tubular system. Calibration line represents 0.4 μ.

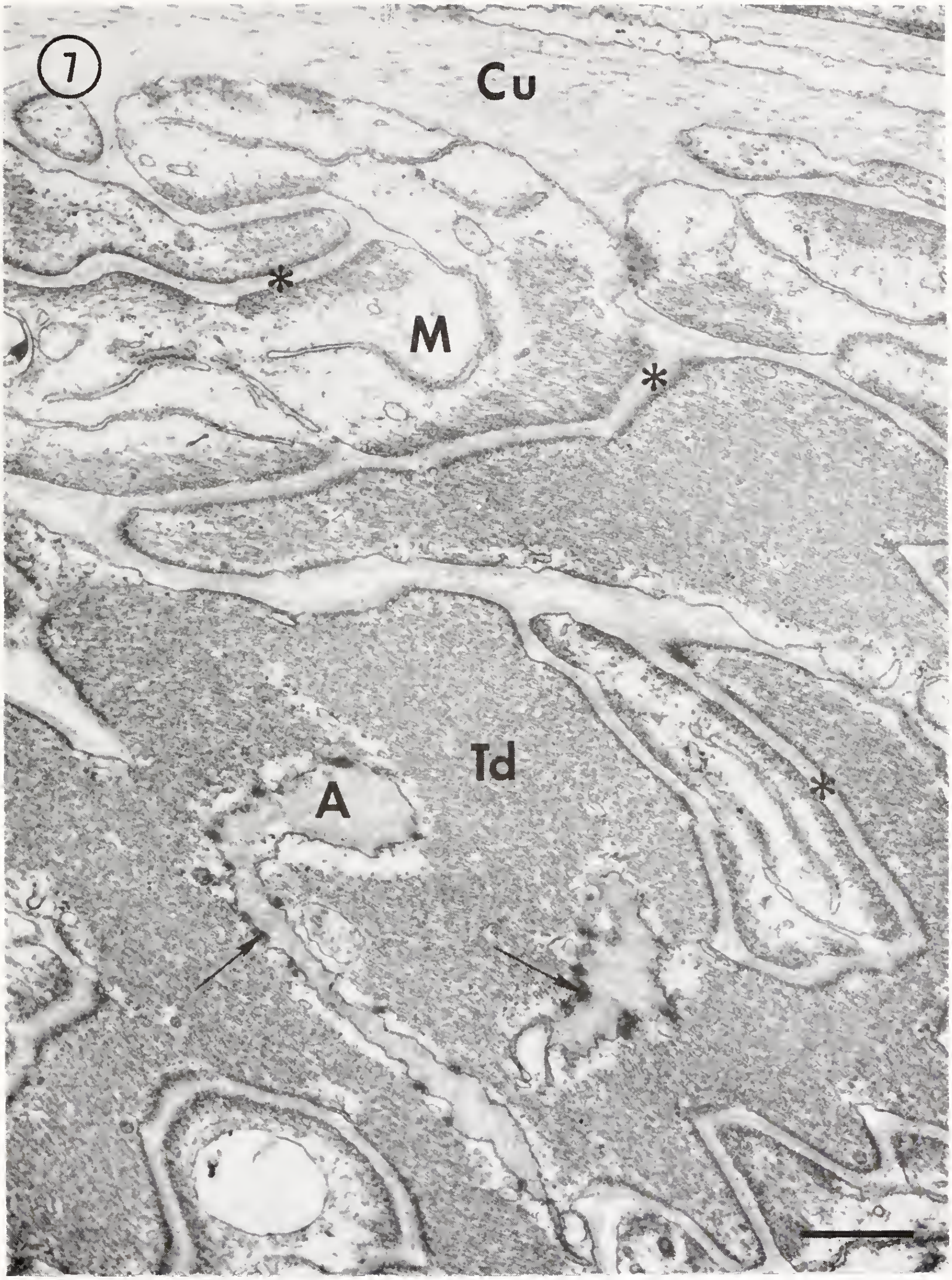

FIGURE 7. Both muscle-tendinal cell and tendinal cell-apodeme attachment sites are shown. The desmosomal-like muscle-tendinal junction (asterisks) shows a very extensive degree of interdigitation between muscle (M) and tendinal-cell (Td). The tendinal cell-apodeme (A) junction is hemidesmosomal (arrows) in nature. Here, dense material lines only the inner surface of the tendinal cell membrane. The attachment is formed along invaginations of the tendinal cell by "tongue-like" extensions of apodeme; Cu, cuticle. Calibration line represents 0.5 μ.

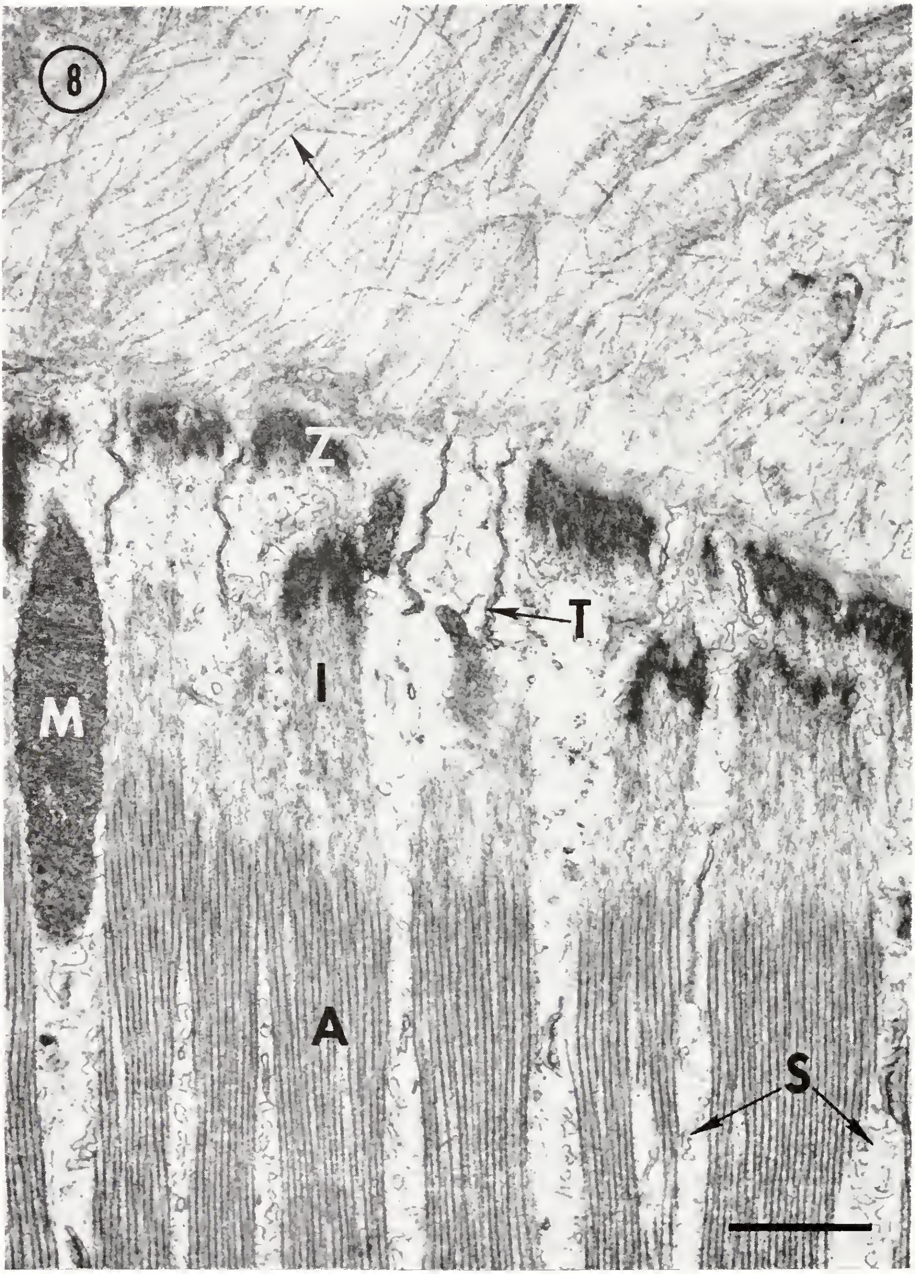

FIGURE 8. Portion of a muscle fiber at its origin showing the attachment of collagen fibrils (unlabeled arrow) to the muscle fiber. The fibrils extend into an amorphous, moderately dense material lining the external aspect of a thickened Z line (Z). The terminal Z line is two to three times the width of "normal" Z lines. This section is oblique, accounting for the

the muscle fiber Z lines. Here, the Z line is 0.2 to 0.4 μ in width, which is about two to three times the normal Z line width. Thin myofilaments join this thickened Z line as they do the Z lines in the muscle fiber interior. A moderately-dense layer of amorphous, basal lamina-like material lies on the extracellular surface of the thickened Z line. The collagen fibrils extend into this material and presumably anchor to it. Although the collagen fibrils are not strictly oriented, in general they lie perpendicular to the long axis of the thickened Z line.

Discussion

The attachment of propodite muscle fibers to skeletal elements in horseshoe crabs is achieved in two ways. At the origin, muscle fibers are attached directly to the cuticle by a network of collagen fibers that extend into the terminal Z lines. Attachment at the insertion is much more elaborate. Here, specialized epidermal cells, the tendinal cells, are interposed between the muscle fibers and apodemes. These elements are connected together in series through desmosomal junctions formed between thin myofilaments and microtubules on the one hand and on the other by hemidesmosomal junctions formed between microtubules and apodemes. In this manner, the contractile filaments are functionally connected to the apodemes, which in many cases are elongated to form tendons (Richards, 1951), by the vast numbers of oriented microtubules in the tendinal cells.

The structural details of the insertion of muscle onto skeletal elements in horseshoe crabs conform to those reported for most of the other arthropod groups. The presence of specialized epidermal cells intervening between muscle and cuticle has been reported for all of the arthropods studied (Lai-Fook, 1967; Caveney, 1969; Smith *et al.*, 1969; Kuo *et al.*, 1971; Koulish, 1973). Desmosomal and hemidesmosomal junctions between muscle and tendinal cell and tendinal cell and cuticle respectively also are typical of arthropods. However, some of the ultrastructural details of the attachment sites differ between the various arthropods.

In insects, in an acarine arachnid and larval stages of a malacostracan crustacean, the tendinal cells are attached to the cuticle by means of narrow elliptical sockets or cone-like depressions invaginated into the tendinal cell. These sockets are connected through hemidesmosomes to extracellular attachment fibers variously referred to as tonofibrillae, muscle attachment fibers and intracuticular fibers which extend into the cuticular layers where they end (Lai-Fook, 1967; Caveney, 1969; Kuo *et al.*, 1971; Talbot *et al.*, 1972). These extracellular fibers are not present in spiders (Smith *et al.*, 1969), scorpions (Mazurkiewicz and Bertke, 1972), barnacles (Koulish, 1973) or in the horseshoe crab material examined in the present study. Thus, the attachment zones in the latter organisms are somewhat simpler in organization.

Another notable difference found in the details of arthropod skeletal muscle insertions occurs in the acarine arachnids (mites). Here, the mechanical force exerted by muscles on the exoskeleton is not transmitted through microtubules, for these subcellular structures are absent from the intervening epidermal cells (Kuo

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apparent double Z line. Features typical of striated muscle are evident; namely, A band (A), I band (I), a mitochondrion (M), sarcoplasmic reticulum (S) and T-tubules (T). Calibration line represents 0.9 μ.

et al., 1971). Instead, only the specialized desmosomal junction is present which apparently is sufficient to provide the necessary intercellular linkage in these very small arthropods.

One particular feature of the desmosomal junctions involved in muscle attachments deserves special mention. The gap between the apposing plasma membranes at the desmosome is considerably wider in many of the arthropods examined than is usually encountered for this type of intercellular junction. The gap width typically is about 250 Å (Farquahar and Palade, 1963), whereas it may reach 700 Å in crustacean muscle attachments (Koulish, 1973), and gaps of 1,000 Å were commonly seen at horseshoe crab muscle insertions. The implications for the mechanical strength of desmosomes having such wide gaps remains to be assessed.

The elaborate extent of the cellular interdigitations at the attachment zones between muscle and tendinal cells and tendinal cells and apodemes results in a considerable increase in the total area available for intercellular mechanical linkage. Lai-Fook (1967) has estimated that the process of interdigitation increases the area of contact at the muscle-tendinal cell junction by a factor of from eight to ten and at the tendinal cell-apodeme junction by a factor of 40. No attempt was made to quantify this feature for horseshoe crab intercellular attachment zones, but a similar extent of interdigitation appears to be present in this organism as well.

The concentration of microtubules in the horseshoe crab tendinal cells is similar to that reported for these cells in an insect (Lai-Fook, 1967) and a spider (Smith *et al.*, 1969). A figure of $750/\mu^2$ was reported in the latter study which is comparable to the concentration seen in horseshoe crabs of $910/\mu^2$. In addition, there is no regular pattern of microtubule distribution in the tendinal cells of these arthropods.

The presence of gap and septate junctions between adjacent tendinal cells has not been reported previously for arthropods. Septate junctions are commonplace in invertebrate epithelial tissues and gap junctions have been found in a variety of tissues in several different animal phyla (*cf.* Hand and Gobel, 1972). A number of functions have been proposed for these specialized junctions. These include intercellular adhesion, intercellular chemical communication and intercellular electrical coupling. Since tendinal cells are primarily involved in the transmission of mechanical forces from muscle to exoskeleton, the tight and septate junctions in all probability provide a strong mechanical linkage between adjacent tendinal cells. Whether or not they provide sites for intercellular communication remains to be determined.

Comparisons between horseshoe crabs and other arthropods in the mode of muscle attachment to exoskeleton at muscle origins cannot be made, since most of the previous studies have not distinguished clearly between muscle origins and insertions. Therefore, it is not known if there are different modes of attachment between origins and insertions in other arthropods. A direct linkage of muscle to cuticle has been reported for a mite, but the precise details of the attachment were not given (Kuo *et al.*, 1971). The muscle responsible for venom expulsion in the black widow spider is attached directly to the fibrous sheath that surrounds the venom gland (Smith *et al.*, 1969). Here, the gland muscle is extensively invaginated by branches of the sheath, and the collagen fibrils in the sheath matrix are thought to provide a mechanical linkage between the myofibrils and the gland.

Summary

At their insertion, propodite flexor muscle fibers in horseshoe crabs are attached to apodemes via specialized epidermal cells, the tendinal cells, which contain tremendous numbers of microtubules. At the desmosomal muscle-tendinal cell junction, thin myofilaments attach to one side of the junction and tendinal cell microtubules to the other side. At the apical end of the tendinal cells, the microtubules extend into the dense material lining the hemidesmosomes formed by the tendinal cells and apodeme. In this manner, thin myofilaments are functionally connected to apodemes through intervening tendinal cell microtubules.

At the muscle origin, collagen fibrils embedded in a matrix connect terminal Z lines, which are two to three times the width of normal Z lines, to the surface cuticle. Tendinal cells and microtubules are not involved, and the attachment of muscle is directly to the exoskeleton.

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BEHAVIOR AND ELECTRICAL ACTIVITY IN THE HYDROZOAN *PROBOSCIDACTYLA FLAVICIRRATA* (BRANDT). I. THE HYDROID COLONY

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During the past decade a number of workers have recorded electrical activity in hydroids while observing overt behavior. The gymnoblasts were *Cordylophora* (Josephson, 1961a, 1961b; Mackie, 1968), *Hydra* (Passano, 1962; Passano and McCullough, 1962, 1963, 1964, 1965; Rushforth, 1965a, 1965b, 1971; Rushforth and Hofman, 1972) and *Tubularia* (Josephson, 1962, 1965a, 1965b; Josephson and Mackie, 1965), and one calyptoblast, *Obelia* (Morin and Cooke, 1971).

Often these animals generate potentials which cannot be correlated with any obvious behavioral events, though occasionally it has been possible to designate one pulse type to a particular movement. Much of the activity has been described as spontaneous with pacemaker control.

Slow, precise postural movements are rarely accompanied by large potentials. If electrical activity can be recorded, at such times, the potentials are usually of short duration with a very low amplitude, often at the limit of the resolving power of the recording equipment (Mackie, 1968). Usually recording has taken place in a homogenous environment where sensory input is presumed to be at a low level.

The conditions under which recordings were taken from *Proboscidactyla flavicirrata* are in marked contrast to the above since *P. flavicirrata* is found only in association with sabellid polychaetes. Many of the recordings were made with the host sabellid in its tube and actively feeding, hence conditions could be regarded as particularly "noisy" (large sensory input) for the hydroid colony.

MATERIALS AND METHODS

P. flavicirrata (Brandt) is a gymnoblastic hydroid found as an obligate symbiont on the outside of the rim of sabellid tubes. The species appears to range throughout the northern coasts of the Pacific, at least as far north as the Queen Charlotte Islands and south to California.

The species found acting as hosts in this study were *Pseudopotamilla ocellata, Schizobranchia insignis* and possibly *Pseudopotamilla reniformis* (only 2 specimens of *P. reniformis* were found infested).

A brief description of the colony of *P. flavicirrata* is given here as it is so dissimilar from most other hydroid colonies. The colony consists of four types of individuals—gastrozooids, dactylozooids, gonozooids, and attached medusae (Fig. 1). Gastrozooids are normally confined to the rim of the tube with their mouths facing towards the axis of the tube. Each gastrozooid bears only two tentacles that arise on the side of the column which faces the host polychaete, just below

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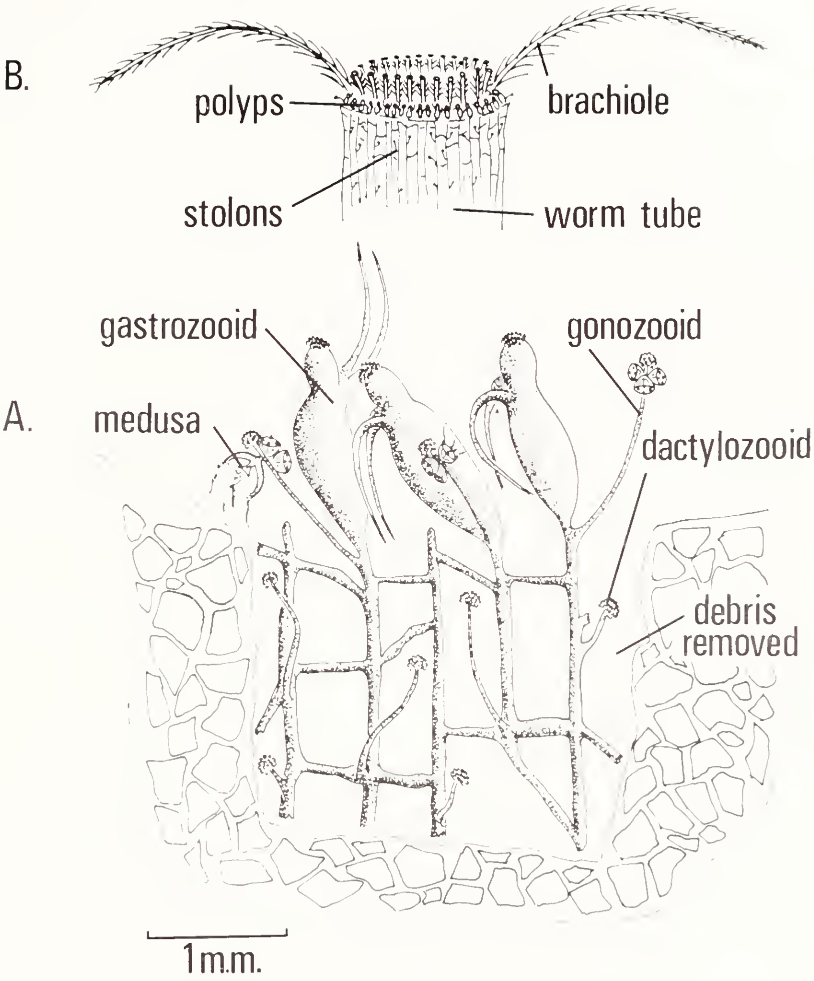

FIGURE 1. (A) Diagram of part of a colony of *P. flavicirrata* with the host worm removed, (B) appearance of the colony with the worm present. Only the proximal portions of most of the brachioles of the worm are shown.

the "neck" of the hypostome. The apex of the hypostome carries a nematocyst pad (cnidothylacie) as do the terminal knobs of dactylozooids and gonozooids. Gonozooids arise close to the bases of the gastrozooids (unless the colony is rapidly advancing as the tube grows). Dactylozooids, which never bear medusa buds, are found further from the rim than gonozooids, usually on longitudinal stolons. In sexual colonies there are eight medusa buds to each gonozooid, each bud being at a different stage in development. In advancing colonies the stolons run parallel at the terminal end of the tube leaving a stationary mat of anastomosing stolons covering proximal portions of the tube. More usually transverse stolons are found at the bases of gastrozooids anastomosing with longitudinal stolons.

Infested sabellids were collected from floats and wharves at the southern end of Vancouver Island and San Juan Island, then transferred to either a non-recirculated natural seawater system (Friday Harbor Laboratories) or a recirculated system (University of Victoria).

In the former system both the hydroids and their hosts obtained an adequate food supply whereas in the latter system the hydroid colonies eventually regressed after a few weeks. Water temperatures ranged from 9 to 13° C and no strict photoperiod was maintained.

Electrical activity was recorded using polyethylene tubing drawn out to a fine tip (20–100 μm), with a length of platinum or Ag/AgCl wire acting as the conductor and sea water as the electrolyte. The electrodes were connected to a syringe so that they could be sucked onto the ectoderm of the hydroid. Ag/AgCl suction electrodes were used to deliver shocks.

The pre-amplifiers of the Grass Model 7 polygraph were used to drive the oscilloscope (Tektronix 502A). Separate capacity-coupled pre-amplifiers were sometimes used with a Gilson Model M5P polygraph. Normally the polygraphs were operated with a differential input, though ground reference recordings were also found necessary. Oscilloscope displays were photographed with a 5-inch Polaroid camera.

Sea water in the recording dish was cooled (11-14° C) by water passing through a glass coil partially sunk into the wax-base of the recording dish.

RESULTS

Behavioral observations

Feeding. The host sabellids feed continuously throughout the day, unless disturbed, when the fan is withdrawn for several minutes. While the fan is erect all three host species continuously create centripetally directed water currents passing through the brachioles of the fan. This current is maintained, presumably for respiratory purposes, even when suspended food material may be scarce. In contrast, gastrozooids feed only when there is abundant food.

A typical microphagous feeding cycle for a single gastrozooid is as follows: (1) The tentacles of the gastrozooid are positioned along the long axis of the food grooves of neighboring brachioles of the sabellid (In most colonies the density of gastrozooids is such as to position individuals between brachioles.)

(2) Both tentacles are extended by relaxation of the longitudinal muscles and elasticity in the mesogloea and possibly the vacuolated endodermal cells (circular muscles are absent (Spencer, 1971)). Tentacles relax by up to 25 times their contracted length.

(3) The tentacles extend down the food grooves towards the oral region of the worm, on occasions they may reach as far as the palps.

(4) The gastrozooids remain in this position for times varying between 10 seconds and several minutes.

(5) Then the tentacles are withdrawn rapidly by either a single contraction or a flurry of contractions of the longitudinal muscle, with flexion in an oral direction. In addition an assymetrical contraction of the longitudinal muscles of the column often occurs, causing the gastrozooid to bend away from the rim.

(6) Usually the tentacles are coated with a mixture of mucus and food particles, and at such times the tentacles are wiped through the mouth singly or together. The mouth gapes, only if food is covering the tentacles (proline at 10^{-5} M induces gaping). The oral hood engulfs the proximal portion of the tentacle first, then the tentacle, in its contracted state, is wiped through the mouth. This stage can take as long as 1 minute.

(7) If food is not present on the tentacles then they are not swallowed and frequently return to Stage 1.

(8) The mouth is closed after both tentacles have been wiped and a slow peristaltic wave passed down the neck pushing food into the gut.

Most of the food gathered in this way consist of unicellular algae and some early larval crustaceans. Nematocysts are not used at such times. In addition to the microphagous feeding cycle described above gastrozooids are capable of capturing and ingesting large prey (*Artemia* nauplii, harpacticoid copepods, nematodes and eggs of the host worm) by using nematocysts. This macrophagous feeding cycle is similar to that described for *Hydra* (Josephson, 1965c).

Behavior while fan is retracted. Immediately following fan withdrawal by the sabellid, feeding often recommences at Stage 5 or 6 for one cycle. When the feeding cycle is completed the tentacles are not extended as would be the case if the fan were erect. However, gastrozooids continue to make abortive feeding movements as in Stage 5 continously until the fan is erected once more. The tentacles are never wiped through the mouth.

The frequency of tentacle contraction and column bending in any one individual decreases from a mean value of 16/ min just after fan withdrawal to about 1 every 15 min if the fan remains withdrawn for several hours. Behavior of gonozooids, dactylozooids and medusa buds is not influenced by the presence or absence of the fan.

Behavior while fan is erect. Fan erection is far slower than fan withdrawal, often taking 30 sec to become fully expanded, whereas withdrawal takes place in less than 1 sec. As the fan slowly emerges from the tube the gastrozooids may rear backwards in response to a wave of colonial retraction (see below), but more usually they relax the tentacles allowing them to trail over the pinnules as they emerge. This is not a prelude to feeding as might be supposed, as tentacles are not swallowed. When the fan is erect gastrozooids go through the feeding cycle as already described.

Protective retraction of the colony and associated responses in the attached medusae. Colonial retraction involves all four types of individuals in the colony and is seen under a variety of circumstances. Withdrawal of the fan by the worm or animals clambering over the colony usually causes colonial retraction. Occasionally colonial retraction occurs when there is no obvious exogenous stimulation.

Basically the response comprises a wave of excitation spreading out from the point of stimulation, involving contraction of all the individuals in the colony. Any portion of the entire surface of the colony, except for a few distant dactylozooids and stolons, can act as a receptor site and set off a wave of contraction. Gastrozooids and tracts of stolon near the rim have lower thresholds than other regions of the colony. Tapping the tube, stolons, gastrozooids or gonozooids with a probe can set off a wave of colonial retraction. As a wave of excitation reaches each individual

TABLE I

Responses of attached medusae to colonial excitation

Size group (μm diam.)	State of tentacles (inside or outside bell cavity)	Response to excitation	Normal activity
0–279	Inside	None*	None*
250–420	Inside	Swim	None
400–490	Inside or outside	Swim or crumple	Swim
490–630	Outside	Crumple	Swimming
630–850	Outside	None	Swimming or crumple

* A response may not have been seen because the young medusa forms a virtually incompressible body at this stage.

the following muscular responses are seen: (1) Gastrozooids, tentacles are contracted synchronously, together with a contraction of the column. (Unlike feeding there is always shortening of the column.) (2) Dactylozooids and gonozooids, muscular activity consists of slight bending and shortening of the terminal quarter of the individual. (3) Medusae, the type of muscular response seen is dependent on the size of the medusa (hence its developmental stage) and is presented in Table I. This table has been condensed from observations made on eight medusae in each size class. The position of the four tentacles is used as an indicator of the developmental stage reached by the medusae. The word "swim" refers to one swimming contraction of the circular muscles. The word "crumple" refers to a contraction of the radial muscles which causes the margin of the jellyfish to be drawn up into the subumbrellar cavity (Hyman, 1940).

Frequently a wave of colonial retraction precedes fan withdrawal, thus preventing feeding gastrozooids from becoming damaged as the brachioles scrape down against the inside of the tube. The precise form of the stimulus initiating colonial retraction, in this case, is difficult to determine. However, if the chaetae of the sabellid are removed from the anterior segment of the worm colonial retraction is only rarely seen preceding fan withdrawal. Colonial retraction then occurs during fan withdrawal. Scraping of the setae and uncinii on the inside of the tube as the worm retreats would seem to be a stimulus capable of setting off a colonial retraction.

Electrical activity

Since gastrozooids dedifferentiate when the worm is removed from the tube it is almost impossible to obtain colonies that have been starved for extended periods. Thus all the recordings are taken from colonies that are, at the worst, poorly fed but certainly not starved. Table II summarizes some of the parameters of the pulse types recorded.

Tentacle contraction pulses (*TCP's*). Tentacle contraction pulses can be recorded from any part of the surface of the tentacles and column of the gastrozooid. They are the electrical accompaniment to tentacle contraction seen during feeding and abortive feeding.

The way in which tentacle contraction pulses can be stimulated artificially fall into three groups: mechanical, electrical and chemical stimulation. Prodding a

Table II

Parameters of the pulse-types

Pulse type	Initial polarity	Duration (msec)	Conduction velocity (cm/sec.)	Amplitude (mV)	
				+	−
Tentacle Contraction Pulses N = 950					
(i) Active tentacle (n = 358)	+	100–440 $\bar{x} = 206$	n.a.	1.0 –3.9 $\bar{x} = 2.6$	0.6 –2.5 $\bar{x} = 1.2$
(ii) Passive tentacle (n = 272)	−	80–160 $\bar{x} = 123$	n.a.	0.07–0.8 $\bar{x} = 0.25$	0.4 –1.9 $\bar{x} = 1.3$
(iii) Column (n = 320)	−	40–230 $\bar{x} = 143$	3–7 (14° C)	0.04–0.8 $\bar{x} = 0.39$	0.24–2.8 $\bar{x} = 1.48$
Column Contraction Pulses N = 54	+	100–420 $\bar{x} = 203$	n.a.	0.5–2.9 $\bar{x} = 2.3$	0.01–1.8 $\bar{x} = 0.5$
Colonial Pulses, N = 782	−	35–130 $\bar{x} = 78$	0.9–9.0 (14° C)	0.23–7.2 $\bar{x} = 1.4$	0.12–3.2 $\bar{x} = 1.3$

tentacle will elicit a single TCP or a burst of such pulses (Fig. 2A), whereas stretching a tentacle causes rhythmical firing (Fig. 2B), the frequency of discharge increasing as the stretching force increases. Single shocks of 1 msec duration delivered to a tentacle evoke a single TCP and never cause multiple firing of TCP's (whatever the stimulus strength).

The only amino/imino acid to cause a noticeable increase in the rate of TCP's was proline (Fig. 3A), which also causes mouth gaping.

Reduced glutathione (a tripeptide) at a final concentration of 10^{-5} M does not elicit tentacle contraction or mouth gaping. However the frequency and duration of TCP's after crustacean tissue fluids are added far exceed those seen after the addition of proline alone (Fig. 3B).

In an intact colony, with the polychaete in its tube, the rate of TCP firing in a gastrozooid is directly correlated to the activity of the worm. While the fan is

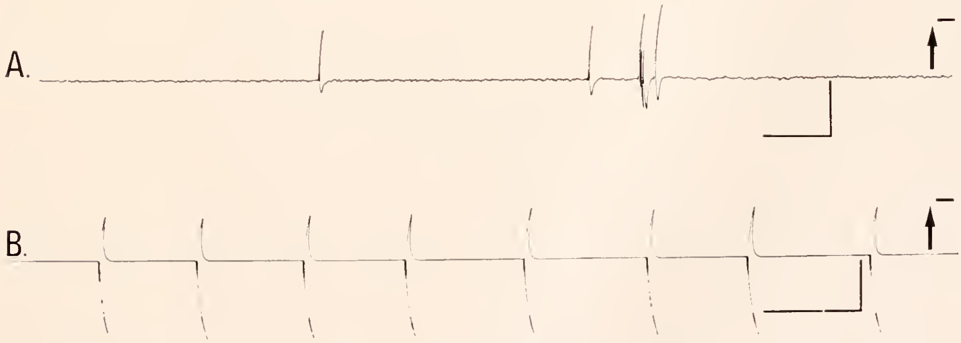

Figure 2. Tentacle contraction pulses recorded from two gastrozooids, (A) a single TCP and burst of TCP's recorded from the column of a gastrozooid after prodding a tentacle. The vertical bar represents 1 mV and the horizontal bar 5 sec; (B) rhythmical firing of TCP's when a tentacle is stretched by sucking it into the recording electrode. The vertical bar represents 1 mV and the horizontal bar, 5 sec.

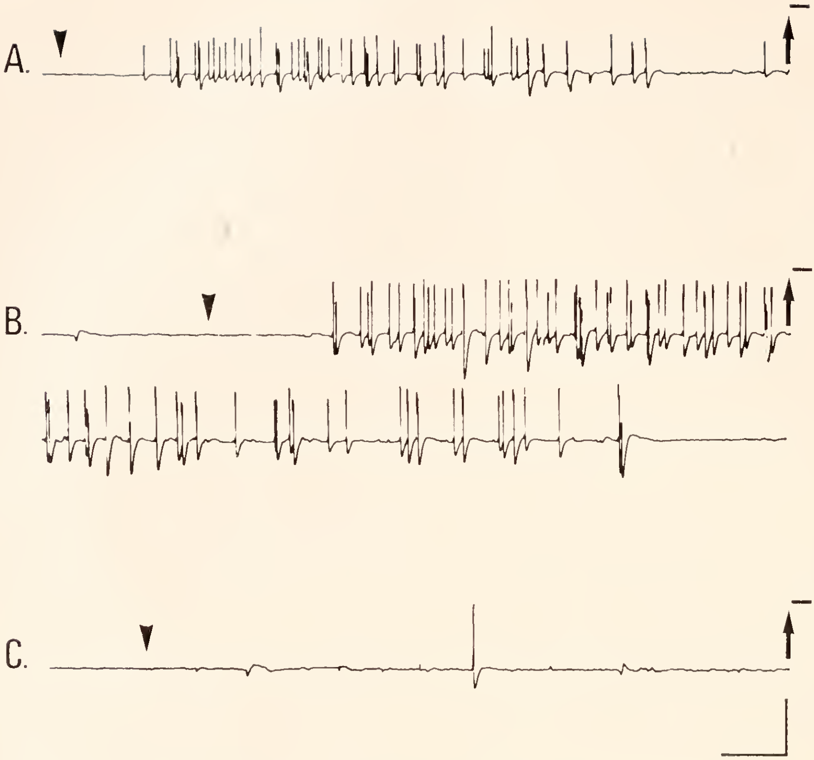

FIGURE 3. Chemical stimulation of tentacle contraction pulses; (A) a burst of TCP's recorded from the column after addition of proline at a final concentration of 10^{-5}M at the arrow; (B) a far longer burst of TCP's after addition of shrimp tissue fluid, also recorded from the column; (C) a single TCP after addition of an equal volume of sea-water at the arrow. Vertical bars represent 1 mV and horizontal bars 5 sec.

erect the rate of TCP firing is fairly constant, 1 pulse or burst every 40 seconds being a typical figure (corresponds to the average duration of a feeding cycle).

If particulate food is scarce the frequency is often higher than this, perhaps 1 pulse every 25 seconds (corresponds to the average duration of an abortive feeding cycle).

Immediately following withdrawal of the fan the frequency of TCP's is high, reaching a rate of 1 pulse every 8 seconds in any one individual. This frequency decreases as the period of fan withdrawal increases until the firing frequency may be as low as 1 pulse every 15 minutes. The rate of TCP firing may increase momentarily during fan erection.

The wave-form of a tentacle contraction pulse differs greatly depending on the recording position. If the recording electrode is positioned on the tentacle which is contracting, then the pulse appears as a biphasic event with the positive-going component being most noticeable (Fig. 4). When the electrode is placed on the other passive tentacle forming a pair then the event appears as a negative-going potential with a small positive overshoot (Fig. 4). Similarly, a TCP recorded

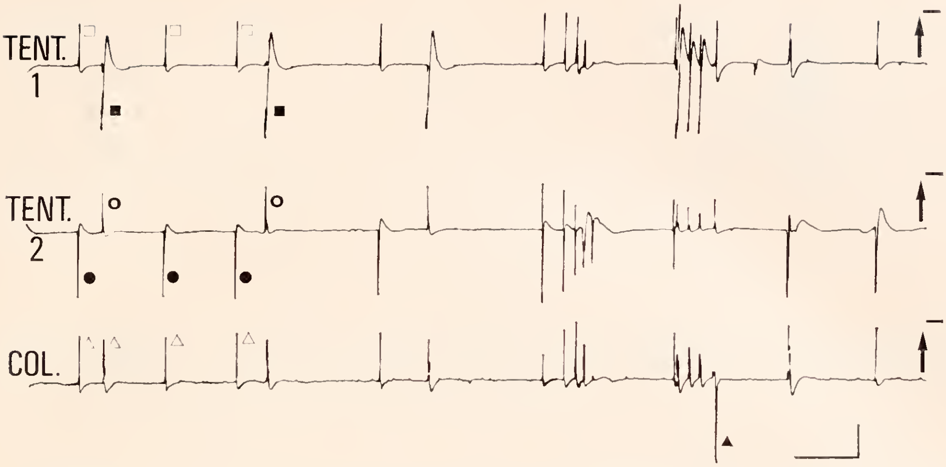

FIGURE 4. One column contraction pulse (CCP) occurring with a number of tentacle contraction pulses (TCP's) recorded from one gastrozooid. One electrode is attached to each tentacle and a third attached to the column. Symbols are: Filled squares, TCP of tentacle 1; filled circles, TCP of tentacle 2; open circles and squares, TCP recorded from passive tentacle; open triangles, TCP recorded from the gastrozooid; and filled triangles, CCP of the gastrozooid. The horizontal bar represents 5 sec and the vertical bar 1 mV.

from the column of a gastrozooid appears as a negative-going potential with a small positive overshoot (Fig. 4).

Conduction velocities of TCP's range from 7.8 cm/sec in the tentacles to 3 cm/sec on the abaxial surface of the gastrozooid measured from individuals partially relaxed, with the tentacles as long as the column (all at 15° C conducted in a distal direction).

Column contraction pulses (CCP's). Column contraction pulses can only be recorded from the column of the gastrozooid. If they are conducted up into the tentacles the amplitude of these pulses must be less than 5 μV. Each CCP corresponds to a contraction of the column longitudinal muscles with fibrils on the abaxial side shortening more than those on the axial side of the column. The frequency of TCP's is always less than that of CCP's.

CCP's can either appear alone or with TCP firing of both tentacles. Conduction velocities of CCP's have not been measured since it is difficult to stimulate CCP's electrically without generating other pulse types, and not enough spontaneous CCP's were recorded to give a statistically reliable value. Figure 4 shows a column contraction pulse appearing in a burst of TCP activity.

Colonial pulses (CP's). Colonial pulses can be recorded by suction electrodes attached to any part of the external surface of the colony (the medusa just prior to release shows no electrical connectivity to the parent colony).

The passage of each colonial pulse through an individual causes protective retraction of that individual as described in the section on behavior. Single monophasic square-wave shocks of 500 μsec, or longer, duration delivered at any position on the surface of the colony will generate a colonial pulse. Multiple firing is rarely seen.

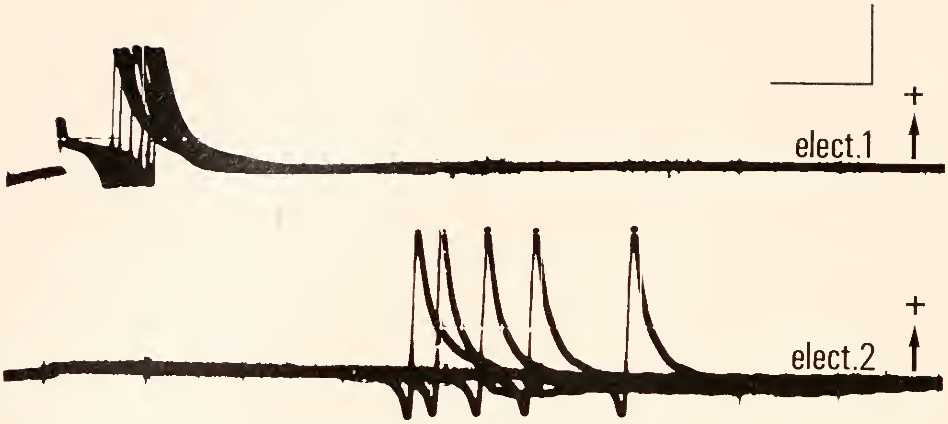

Figure 5. Superimposed oscilliscope traces of repetitively stimulated colonial pulses (1 pulse every 2 sec) recorded from the bases of two widely separated gastrozooids. Six shocks were delivered on a stolon close to electrode 1 which recorded six colonial pulses. Each successive colonial pulse was conducted at a lower velocity than its predecessor with the last pulse failing to arrive at electrode 2. The horizontal bar represents 50 msec and the vertical bar 1 mV.

There is evidence that the phenomenon of facilitation does exist in the colonial pulse conducting system, but such evidence is not consistent. For example, in one colony the first shock delivered to the colony at the base of a gastrozooid may fail to elicit a CP, but the second shock delivered 2 sec later (at the same voltage) does set off a colonial pulse. Two hours later, with the same preparation, the first shock gives a colonial pulse.

Usually a colonial pulse generated by the first shock is conducted throughout the colony. Occasionally the first CP is not conducted to the furthest part of the colony, whereas the second and third CP's stimulated within a few seconds do reach previously unexcited areas. After perhaps twenty pulses, the distance through which each pulse conducts begins to decrease, until suddenly the whole colony becomes refractory to further excitation.

Since the stolonal network is so complex, with numerous cross connections, the distance a CP must travel from the stimulating site to the recording electrode is difficult to measure. The number of alternate routes runs into many thousands.

It must be remembered that a CP travels as a wave of excitation eventually affecting all parts of the colony, thus many stolons are transmitting the same colonial pulse simultaneously.

Conduction velocities of CP's measured from long tracts of stolon using two recording electrodes give a mean value of 1.9 cm/sec (13° C, $N = 45$) with a minimum value of 0.9 cm/sec (13° C). In comparison measurements from short lengths of stolon without any cross connections or sharp bends give a mean value of 7.3 cm/sec (13° C, $N = 38$) with a maximum value of 9.0 cm/sec (13° C). These differences are presumably due to an inability to correctly measure the conduction distance in long tracts, they being considerably underestimated.

All parts of the stolonal network are unpolarized as regards both conduction

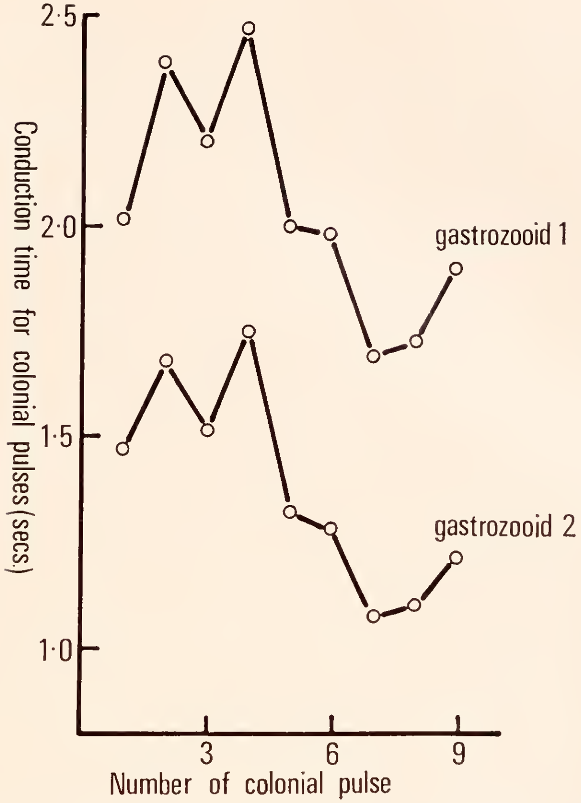

FIGURE 6. Graph to show that fatigue and recovery from fatigue of the colonial pulse system affects all parts of the colony equally. Nine shocks were given randomly to the colony over a period of one minute at a distant stimulating electrode. The time taken for each colonial pulse to reach two gastrozooids is shown.

velocity and facilitation. Conduction velocities are not significantly higher in large diameter stolons.

The conducting system for colonial pulses is particularly labile. At a constant stimulation rate of 1 shock/sec the system gives one CP for each shock for between 5 and 60 shocks, then follow periods of firing to alternate shocks, every third, fourth, fifth and sixth shock until the system no longer conducts. This effect can be brought about more rapidly by increasing the rate of stimulation. If a colony is stimulated with repetitive shocks having a frequency greater than 1 every 5 sec, then the conduction velocity of each colonial pulse decreases noticeably until the conducting system becomes inexcitable (Fig. 5). Figure 6 shows that the rate of fatiguing and recovery from fatigue affects all parts of the colony equally. As might be expected the threshold for generating a colonial pulse increases as the preparation fatigues.

Colonial pulses continue to be propagated after the colony has been immersed in a solution containing 1 part of sea-water to 1 part of isotonic magnesium chloride for three hours.

Discussion

It is not surprising that the earliest accounts of the hydroid phase of *Proboscidactyla* include graphic description of the tentacle contraction and column bending at Stage 5 of the feeding cycle, since these movements are immediately obvious (Gosse, 1857; Hincks, 1872). However these authors only hint at the functional significance of such movements.

Even the most recent account of feeding (Hand and Hendrickson, 1950) fails to mention tentacle swallowing. These authors believe that back-bending positions the tentacles at right angles to the incoming water current, enabling gastrozooids to catch particles in this stream. Unfortunately, they do not describe how the food material is ingested.

Why does the genus *Proboscidactyla* have only two tentacles? If *Proboscidactyla* is not essentially a predatory hydroid then only two possibilities remain. Either it could be feeding on suspended, non-motile food as it is carried across the branchial crown of the sabellid, or it could be taking this food after it has been concentrated in the food-grooves of the host sabellid. The first possibility seems unlikely since it is an accepted principle that suspension feeders present a large collecting surface to the food-carrying current. Indeed the observations made in this study support the proposition that *Proboscidactyla* could be treated as a deposit-feeder despite the parasitic character.

Proline, rather than reduced glutathione, may well be the most important substance controlling feeding in the marine and brackish-water gymnoblastic hydroids, since *Cordylophora* and *Pennaria* as well as *Proboscidactyla* give a feeding response to proline, but not to reduced glutathione (Fulton, 1963; Lenhoff, Muscatine and Davis, 1968).

There is no doubt that *P. flavicirrata* consumes a considerable quantity of plant material (which may or may not be utilized). This is a habit not frequently met with in the Cnidaria.

A number of colonial hydroids show propagated retraction of polyps when mechanically stimulated (Wright, 1856; Zoja, 1891; Josephson, 1961a). The muscular responses seen in *P. flavicirrata* most resemble those seen in *Hydractinia* (Josephson, 1961a). Feeding polyps in both species contract while the defensive individuals (spiral zooids and dactylozooids) wave about.

The responses of attached medusae to colonial excitation have not been previously described. Varying responses of medusae to colonial excitation is predictable if one considers the tissue connections of the medusa-bud with the colony. Size group 400–490 μm has both an ectodermal and endodermal connection with the colony and responds to a colonial pulse by either swimming or crumpling. In medusae of 490–630 μm diameter the endoderm has been nipped off and the medusae can only respond by crumpling. Just prior to release only a mesogloeal neck remains and neither swimming or crumpling can be elicited by a colonial pulse. From what is known of swimming and crumpling in the mature medusa and the conducting routes responsible for such behavior (Spencer, 1971), it seems probable that the ectoderm is conducting the colonial pulse so as to cause crumpling whilst a colonial pulse conducted in the endoderm causes swimming. Where both ectoderm- and endodermal connections are present between the gonozooid and medusa a CP can cause either swimming or crumpling, with swimming being the common response in younger attached medusae.

Most of the hydroid species so far used for electrophysiological investigations tend to become electrically silent when starved (Passano and McCullough, 1964; Mackie, cited by Josephson, 1965b; Mackie, 1968). From what is known of the biology of *P. flavicirrata* it is very unlikely that colonies ever experience extended periods of starvation. Poorly fed colonies can be recognized in the laboratory, but at no time were obvious differences in the electrical activity of such colonies noticed, as compared to well-fed colonies.

Since every TCP is accompanied by muscular contraction, and the frequency of TCP's is controlled, to a large extent, by exogenous events then the system generating these potentials cannot be called pacemaker-like by the definitions that have come to be accepted by coelenterate electrophysiologists. This does not deny the possibility of some underlying pacemaker control of TCP firing. Judged from the very different wave-form of TCP's recorded from regions where muscle is contracting and passive neighboring areas, TCP's are likely to be compounded from epithelial excitation and electrical events associated with excitation-contraction (muscle potentials). Thus a TCP recorded from a tentacle which does not contract would be an epithelial potential with perhaps some contribution from underlying nerve elements.

Comparisons can be made, if somewhat cautiously, between tentacle contraction pulses and similar potentials in other hydroids. Where rhythmical potentials, associated with muscular activity in tentacles, have been recorded in other hydroids a pacemaker system or systems is always tied in with these rhythmical potentials. For example, in *Tubularia* tentacle potentials accompany oral flexion of tentacles with no synchrony between tentacles except during hydranth pulse bursts (Josephson and Mackie, 1965). In *Hydra* column contraction pulses (also involving tentacle contraction) occur in bursts with a predictable rhythmicity that is dependent on such factors as light intensity, feeding, mechanical stimulation and a pacemaker system, the rhythmic potentials (Passano, 1962; Passano and McCullough, 1962, 1964).

The observation that TCP's can be set off by stretching a tentacle suggest that either the epithelio-muscular cells can be excited by stretching or that the tentacles contain stretch receptors. This has already been suggested for the chondrophoran *Porpita* by Mackie (1959).

Under conditions where it can be expected that TCP's will be generated by excitation of sensory cells, TCP's appear as bursts; for example after mechanical and chemical stimulation. If, on the other hand, the intervention of sensory cells is bypassed then single TCP's are generated after giving above threshold shocks. There is no evidence of neuro-muscular facilitation in the TCP conducting system, one supra-threshold shock always causes contraction. Even in anemones where neuro-muscular facilitation has been clearly demonstrated (Pantin, 1935a, 1935d) tentacles respond earlier in a stimulus burst than does the sphincter (Josephson, 1966).

In no other hydroid has an electrical event, been recorded with so much control over its frequency coming from outside the animal. This extrinsic control appears to dominate any underlying endogenous rhythm. It is only during periods when the fan is retracted that the TCP frequency may settle down to a fairly constant level with no obvious exogenous stimulation. Much of the control of TCP firing therefore comes from the host worm which is to be expected in a relationship where the hydroid is a dependent. It is of great advantage to the colony that its behavior be closely related with the host's, particularly when both are feeding.

The frequency of column contraction pulses is controlled by the same factors as TCP's except that proline and crustacean tissue fluid does not increase the rate of firing. Indeed the recordings of TCP bursts after addition of feeding activators never include CCP's. This could indicate that the receptors are located in the tentacles and/or that excitation from the chemoreceptors is not conducted to the column musculature. Rushforth, Krohn and Brown (1964) have shown that the receptors of G.S.H. in *Hydra* predominate in the tentacles.

The occurrence of TCP's in both tentacles and with CCP's is rarely synchronized, except of course after colonial pulse excitation. It seems that simultaneous contraction of the longitudinal musculature in both tentacles, perhaps with column contraction, is a chance happening. These three muscle systems must either be controlled by separate pacemakers or, as seems more likely, by separate sensory systems. What is also surprising is that excitation of one muscle system does not cause contraction in neighboring systems even though large potentials are being conducted by cells in these regions, for example TCP's recorded from a passive tentacle.

For many years now coelenterate conduction systems have been classified into two broad categories, through-conducting systems and facilitating systems (Pantin, 1935a, 1935b, 1935c, 1935d; Josephson, 1965b, 1965c). Any attempt to fit the colonial pulse system of *P. flavicirrata* into either of these categories fails. In most preparations conduction of colonial pulses meets the requirements of a through-conducting system, but other preparations show incremental properties with successive pulses travelling greater distances through the colony. Similarly the system can also be decremental in respect to conduction distances, velocities and its refractoriness with fatigue.

Within the Hydroidea the Hydractinidae possess colonial co-ordinating systems that most closely resemble that found in *P. flavicirrata* (Josephson, 1961a). Josephson's studies show that gymnoblastic hydroids not belonging to these groups do not possess all-or-none conducting systems that give colony wide excitation to the first shock. It should be noted that in *Hydractinia echinata* the colonial system can facilitate, but this is unusual. Within the corals only the perforate madreporaria give colonial retraction to the first shock with additional shocks facilitating the spread of excitation, except *Acropora* (Horridge, 1957).

Of the facilitating types of systems that found in *Cordylophora lacustris* has been most carefully studied (Josephson, 1961b). Electrical stimulation of the stolon of a *Cordylophora* colony causes bursting of pulses, the number of pulses and their amplitude facilitating with repetitive stimulation and the intensity of the shock. The greater the number of pulses the greater and faster is contraction of the polyp nearest the recording electrode. Also the number of pulses in each burst determines the distance that the excitation spreads through the colony. At no time did *Proboscidactyla* give multiple colonial pulses to a shock. One other colonial cnidarian should be considered, the siphonophore *Nanomia cara*. This animal is capable of a defensive motor pattern that causes stem contraction and synchronized, reserved swimming in the nectophores (Mackie, 1964). The excitation is conducted by ectodermal tissues, at least in parts of the colony, and can be elicited by a single shock. *Nanomia,* however, has evolved a more sophisticated conducting system than has been found in any other cnidarian which involves the use of gigantic syncytial cells in the stem (Mackie, unpublished observations) enabling high conduction velocities upto 100 cm/sec (14° C) (Mackie, 1964).

It seems likely that neurons and muscular elements are absent from the stolons of *P. flavicirrata* (Spencer, 1971) and thus the spread of colonial pulses through the stolonal network is a purely epithelial event. Within the individuals of the colony however nerve and muscle cells may contribute to the spread of excitation. Observations on the responses of attached medusae to colonial pulses as they mature and are finally released indicate that both the ectoderm and endoderm are involved in conducting colonial pulses within the stolons and gastrozooids. The fact that CP's continue to be propagated after exposure to excess Mg^{++} also suggests that conduction in this system is non-nervous since magnesium ions are known to have an inhibitory effect on the appearance of miniature potentials at the post-synaptic side of chemical junctions (Katz, 1962). Other hydrozoan (*Sarsia, Euphysa, Nanomia*) epithelial conducting systems are capable of functioning in the presence of excess Mg^{++} while supposed neuronal pacemakers and neuromuscular events are blocked (Mackie, 1964; Mackie and Passano, 1968). However this does not mean that susceptibility to magnesium anaesthesia can be used to unequivocally distinguish nervous from non-nervous conducting systems, as Mackie and Passano (1968) were careful to point out.

That recent studies of epithelial conducting systems in the cnidaria have not revealed the occurrence of velocity changes that depend on the rate of stimulation is remarkable. McFarlane (1969) may have recorded similar events in *Calliactis parasitica.* He showed that in the column of *Calliactis* there is a slow conducting system (SS_1) that is probably epithelial. Using just one recording electrode he demonstrated that the time taken for each potential of the SS_1 to reach the recording electrode increases with repetitive stimulation if the stimulating frequency is beween one shock/3 sec and one shock/20 sec. At these rates the system eventually fails to conduct. McFarlane interprets these results as being due to an increase in the delay taken to generate a pulse at the stimulating site. Unfortunately he did not test the possibility that the increased delay in arrival of each pulse at the recording electrode may be mostly due to a decrease in conduction velocity rather than increased generation time.

A similar fatigue phenomenon to that seen in *Proboscidactyla* has been reported recently in chick cardiac muscle where repetitive stimulation of slow conducting regions caused a 2:1 block at 4 beats/sec which was preceded by a cyclic, progressive slowing of conduction (Lieberman, Kootsey, Johnson and Sawanobori, 1973). A full discussion of the likely mechanisms of such fatiguing in the colonial pulse system will be included with an analysis of a similar epithelial event described in a following paper on the medusa of *Proboscidactyla flavicirrata.*

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SUMMARY

1. *P. flavicirrata* feeds on material collected from the brachiolar food-grooves of the host sabellid worm.

2. The feeding cycle involves wiping the tentacles through the mouth. An abortive feeding cycle can also be seen when the tentacles are not wiped through the mouth.

3. The frequency of the feeding cycle is in part controlled by activities of the host worm.

4. Tentacle contraction pulses and column contraction pulses are the electrical accompaniment to feeding and abortive feeding. Their properties are described.

5. Colonial pulses, conducted in part by epithelial cells at velocities between 1.9 and 7.3 cm/sec (13° C), cause protective retraction of all the individuals in the colony. The varying response of medusa buds as they develop is described. The colonial pulse conducting system fatigues rapidly after repetitive stimulation.

6. Colonial pulses continue to propagate after exposure to excess Mg^{++} for 3 hr.

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THE CHOROID RETE MIRABILE OF THE FISH EYE. I. OXYGEN SECRETION AND STRUCTURE: COMPARISON WITH THE SWIMBLADDER RETE MIRABILE[1]

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Vascular counter-current systems in which the vessels are of capillary dimensions have been described for the mammalian kidney, the swimbladder of teleost fish (Woodland, 1911; Harden Jones and Marshall, 1953), and the choroid layer subtending the retina of the fish eye. Albers (1806) was the first to show that the large, horseshoe-shaped body in the choroidal layer of the eye of fish was neither a muscle nor a gland as was then supposed but an aggregation of small blood vessels. These vessels originate from a branch of the ophthalmic artery and their outflow supplies the choriocapillaris, the dense capillary bed underlying the retina (Müller, 1839; Jones, 1838). Müller (1839) pointed out that all the blood to the choroidal vessels had first to pass through the pseudobranch. Allen (1905) injected the vessels of the choroidal circulation and later (1949) gave a detailed account of them. He pointed out that the blood flowing to the eye in the ophthalmic artery is oxygenated at the gills and, in many species, again comes in contact with sea water at the pseudobranch. Jones (1838) introduced the now preferred name for the choroidal vascular structure—the choroid rete mirabile.

Barnett (1951) gives by far the most detailed description of the choroidal circulation. It is based on dissection of injected preparations and on serial sections of whole eyeballs or whole heads. Johannes Müller (1839), Richard Owen (1836) and T. Wharton Jones (1838) had each recognized that the arterial and venous capillaries making up the choroid rete mirabile are arrayed in parallel. It remained for Barnett (1951) to realize that the arterial and venous blood streams flow counter-current one to another in the capillaries of the choroid rete mirabile. In addition to the choroid rete mirabile, Barnett (1951) describes a similar, smaller structure, the lentiform body, also supplying blood to the choriocapillaris.

It appeared to us that the pigment cell epithelial layer of the retina is at least formally analogous to the gas gland of the swimbladder (Wittenberg and Wittenberg, 1962). These relations are diagrammed in Figure 1. In each instance a capillary counter-current organ, a rete mirabile, supplies arterial blood to a capillary network, underlying an epithelial layer, respectively the pigment cell layer of the retina and the gas gland of the swimbladder, and in turn receives the venous outflow from these capillaries. The structural resemblance between the swimbladder

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and the choroid retia suggest to us that their functions may be similar, and that the choroid rete mirabile-pigment cell layer complex may serve to create a large pressure of dissolved oxygen behind the retina, providing a pressure for diffusion to supply the vigorous oxygen demand of the avascular retina. We searched for and found large oxygen pressures in the vitreous humor, near the retina of the fish eye (Wittenberg and Wittenberg, 1962), an observation confirmed by Fairbanks, Hoffert and Fromm (1969).

In the present communication we present a more full account of these findings, and examine quantitatively the way in which the structure of the choroid rete mirabile is adapted to provide a large flow of oxygen toward the retina. We describe the structure of the choroid rete mirabile of the holostean, *Amia calva,* and amplify Barnett's (1951) description of the choroid rete of teleosts. Elsewhere the distribution of the choroid rete mirabile among fishes and its relation to the pseudobranch is considered (Wittenberg and Haedrich, 1974).

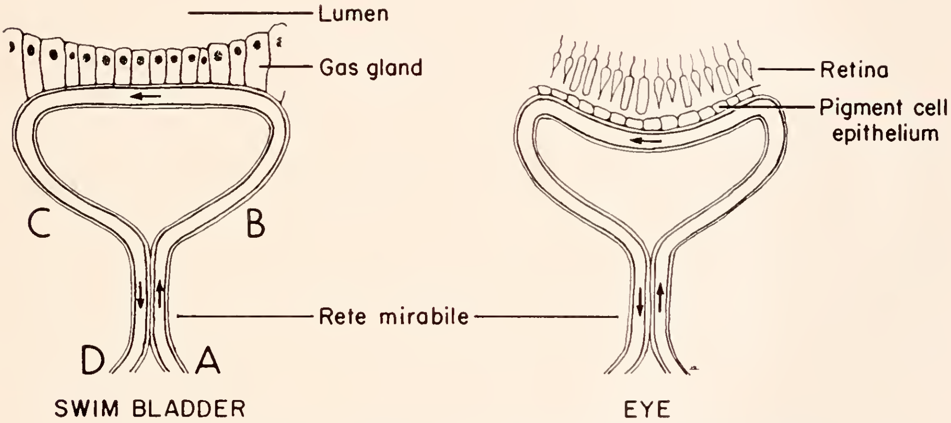

FIGURE 1. Diagram comparing the oxygen-secreting complexes of the eye and the swimbladder of fish.

MATERIALS AND METHODS

Animals

Most of the marine fish used in this study were captured in a fish trap maintained by the Marine Biological Laboratories, Woods Hole, Massachusetts. Some were caught in pots or on hand lines. Cod were the gift of Mr. Charles Wheeler, Bureau of Fisheries, Woods Hole, Massachusetts. Specimens of *Amia* (*Amia calva*) (used for anatomical studies) were the gift of Mr. William A. Lemberger, Oshkosh, Wisconsin. Specimens of *Amia* and gars (Lepisostidae) (used for measurement of ocular pO_2) were captured by Dr. D. Eugene Copeland, Tulane University, New Orleans. Anatomical studies were made of the eyes of cod (*Gadus morhua*), bluefish (*Pomatomus saltatrix*) and of the holostean, *Amia calva.*

Histology and injection preparations

Histological sections were prepared from eyes fixed in Bouin's solution. Blood vessels were injected through the ophthalmic artery or vein with Indian ink in 7% gelatin, with yellow lead chromate in 7% gelatin, or with neoprene latex. Injected preparations were fixed in acid formaldehyde (formalin, 10 volumes, glacial acetic acid, 5 volumes, water to 100 volumes) and were transferred to 70% ethanol for dissection or were cleared and dissected in benzyl benzoate.

Measurements of dimensions of the retia or of intraretinal distances were made with a calibrated ocular micrometer. All measurements are referred to tissue fixed in Bouin's solution and measured in 70% ethanol. Measurements of histological sections are corrected for 22% linear shrinkage. Each set of data given in Table IV is for an individual animal. An additional specimen of each species was examined with similar results. The eels were of average size, 1–2 lb; the conger was of modest size, about 10 lb; the swordfish weighed 160 lb dressed, which is not large; and bluefin tuna weighed about 150 lb.

Oxygen pressure in the eye

Oxygen pressure was measured with an oxygen sensing electrode mounted in the tip of an 18-gauge hypodermic needle (Beckman Spinco Division, No. 161–950 Oxygen micro-electrode). The electrode current was measured with a Keithly model 600A, battery-operated electrometer, or with a Beckman model 160 oxygen analyzer. Calibration was with air equilibrated water and with a solution of zero oxygen pressure (buffered 5% glucose containing glucose oxidase and catalase).

The electrode was introduced into the front of the eye passing between the lens and iris into the vitreous humor. Most measurements were made with the sensing tip of the electrode in the vitreous humor immediately in front of the retina.

Measurements of tissue oxygen pressure made with oxygen electrodes are subject to a number of errors due to inhomogeneity of the tissue and occlusion of capillary blood flow at the electrode tip. The measurements reported here are free from these difficulties. The readings were stable with time and, since the diffusion coefficient of oxygen in a material as fluid as the vitreous humor is scarcely different from that in water, should correspond closely to the true oxygen pressure. If the tip of the electrode chanced to be pushed against the retina, the apparent oxygen pressure dropped immediately, suggesting that the circulation was occluded locally. Further validation of the use of the oxygen electrode comes from the work of Jacobi and Driest (1965), who found, as expected, that the measured oxygen pressure of the vitreous of the rabbit eye increased as the electrode was moved closer to the source of the oxygen at the vascular retina.

Measurements on marine fish were made on living fish with a current of sea water passing over the gills. Measurements on the freshwater fish, gars and *Amia*, were made on living animals restrained and submerged in the water of the bayou.

Gars (spotted gar, *Lepisosteus oculatus* and alligator gar, *L. spatula*) and *Amia* were seined from a shallow bayou near Lake Penchant in the Mississippi River delta. Measurements were made immediately as the fish were brought to the surface so that measurements on gars and on *Amia* are interspersed. During the

TABLE I

Oxygen partial pressure in the eyes of fish

Species		No. of animals	Oxygen pressure Average (range) (mm mercury)	Rete
Marine fish				
Sting ray	*Dasyatus centroura*	2	16 (7–34)	Absent
Skate	*Raja ocellata*	3	10 (6–17)	Absent
Smooth dogfish	*Mustelus canis*	3	11 (9–11)	Absent
Eel	*Anguilla rostrata*	2	18 (9–23)	Absent
Conger	*Conger oceanica*	4	8 (2–12)	Absent
Toadfish	*Opsanus tau*	4	18 (6–28)	Minute
Goosefish	*Lophius americanus*	4	93 (39–146)	Minute
Sea bass	*Centropristes striatus*	2	161 (130–180)	Small
Tautog	*Tautoga onitis*	1	210	Small
Sea robin	*Prionotus carolinus*	3	468 (280–860)	Large
Fluke	*Paralichthys dentatus*	1	255	Large
Puffer	*Sphaeroides maculatus*	2	462 (365–575)	Large
Scup, porgy	*Stenotomus versicolor*	7	484 (272–770)	Large
Menhaden	*Brevoortia tyrannus*	2	255 (240–287)	Large
Goggle eye jack	*Trachurops crumenophthalmus*	4	416 (317–566)	Large
Cod	*Gadus morhua*	3	820 (575–1180)	Large
Bluefish	*Pomatomus saltatrix*	4	454 (240–820)	Very large
Remora	*Echeneis naucrates*	3	775 (435–1320)	Very large
Freshwater fish				
Trout	*Salmo gairdneri*	16	445 ± 68.5*	Large
Gar	*Lepisosteus* sp.	5	90 (72–145)	Absent
Bowfin	*Amia calva*	14	200–650**	Large

* Data of Fairbanks, Hoffert and Fromm (1969).

** See text and Table II.

course of the day the temperature of the water near the surface rose from about 22° C to about 33° C. The bottom water, from which the fish were taken, remained cool. This can introduce an artifact in the determination of pO_2, since at constant oxygen content, pO_2 increases with the temperature of the solution (in this instance the vitreous humor). The maximal effect of this artifact, for the temperature interval 22–33° C, is to increase the apparent pO_2 by 20%. An internal control of this artifact is comparison of the pO_2 in the eye of *Amia* with that of gars, which do not have an elevated oxygen pressure in the eye, measured in the same series of experiments. A second source of error is the small size of the eye of *Amia* relative to the electrode (which was the smallest available at the time, 1963, the experiments were done). Many times the apparent initial pO_2 in the eyes of *Amia* was large but drifted downward before a stable reading was obtained. This effect was not seen with gars, in which the eye is larger. The data are presented in Table II. Only stable readings were recorded in the table; they are not corrected for the possible temperature artifact.

Measurements on warm-blooded animals were made in a 40° C room, so that the electrode temperature differed only slightly from that of the animal. Cats were anesthetized with nembutal, 30 mg per kilogram, administered intraperitoneally. Rabbits were anesthetized with urethane, delivered slowly into the ear vein until deep anesthesia was achieved. Birds were anesthetized with ether. The animals were sacrificed without recovery from anesthesia.

RESULTS AND DISCUSSION

Very large pressures of oxygen were found in the eyes of some fish, Table I. A variety of bottom-living marine fishes, sea robins, fluke, puffer and scup and two pelagic fishes, jack and menhaden, exhibited oxygen pressures in the vitreous humor ranging from 250 to 800 mm Hg. The oxygen pressure in the eye of trout living in fresh water (data of Fairbanks *et al.*, 1969) falls within this range. These pressures should be compared to the oxygen pressure of arterial blood, which is presumably close to that of the ambient water, 155 mm Hg, and in trout is 85 mm Hg (Stevens and Randall, 1967). Three species, cod, bluefish and remora, deserve special comment. The choroid rete of the last two of these fast-swimming, sight dependent, predaceous animals is so greatly developed that the rear of the eyeball bulges asymmetrically to accommodate it. The rete of the third predator, cod, is large but symmetric. The bluefish tolerated handling poorly, and the measurements of oxygen pressure in the eye of this species were at best erratic. The remora and the cod, by contrast, are tough and resistant to laboratory insult. In two individual remora and one cod we measured oxygen pressures of 1000, 1300 and 1180 mm Hg respectively, pressures greatly in excess of one atmosphere (760 mm Hg).

TABLE II

Oxygen pressure in the eyes of individual gars and Amia.

Species	Oxygen pressure Right eye	Left eye
	mm Hg	
Spotted gar *Lepisosteus oculatus*	87	145
	80	102
	—	75
Alligator gar *Lepisosteus spatula*	72	80
	80	—
Bowfin *Amia calva*	204	—
	167	180
	220	—
	62	1210
	440	—
	400	136
	240	235
	100	180
	390	300
	—	220
	—	590
	104	770
	845	—
	—	115

We were led to perform these experiments by the postulate that the choroid rete mirabile is concerned with oxygen transport. This postulate may be evaluated by comparison of species in which the rete is small or lacking with those in which it is large. We have examined six species which lack choroid retia. Of these two, eel and conger are teleosts; three, sting ray, skate and smooth dogfish, are elasmobranchs; and one, the gar, is a holostean. All exhibit low oxygen pressures (10–30 mm Hg) in the vitreous humor. The goosefish and toadfish depend in part on chemical or tactile senses to find their food. The choroid rete of these species is minute, and the oxygen pressure in the vitreous is correspondingly low, scarcely exceeding the probably venous oxygen pressure. Two bottom-living teleosts, sea bass and tautog, have relatively small choroid retia and exhibit oxygen pressures in the vitreous not too different from air. Teleosts with larger choroid retia exhibit correspondingly larger oxygen pressures in the vitreous. The observed correlation between the measured oxygen pressure and the extent to which the choroid rete is developed suggests that the rete plays an essential part in establishing the large oxygen pressure at the retina.

TABLE III

Oxygen pressure in eyes of some vertebrates

Species	Number of animals	Oxygen pressure Average (range)
		mm Hg
Bullfrog *Rana catesbiana*	3	41 (33–59)
Alligator *Alligator mississipiensis*	1	42
Turtle *Pseudemys floridana*	3	41 (37–47)
Turtle *Graptemys pseudogeographica*	2	29 (25–34)
Rabbit	4	16 (7–31)
Rabbit	–	— (17–33)*
Cat	3	19 (15–22)
Cat	–	19**
Pigeon	5	50 (32–64)
Chicken	3	48 (40–53)
Duck	1	44

* Data of Jacobi and Driest (1965).
** Data of Alm and Bill (1972).

The choroid rete mirabile of the holostean, *Amia calva,* the only non-teleost found to have a rete (Wittenberg and Haedrich, 1974), resembles that of teleosts. The rete of *Amia* may represent an independent evolutionary development convergent with that of teleosts; or teleosts and *Amia* may share an immediate common ancestor. This at present is a matter of debate (Nelson, 1969a, 1969b). For this reason it was of particular interest to establish whether the oxygen pressure in the eye of *Amia* is elevated. This measurement was made with some difficulty and an average normal value cannot be given. Nonetheless the data assembled in Table II leave no doubt that a large oxygen pressure obtains in the *Amia* eye. We conclude that the choroid retia of *Amia* and teleosts serve the same function—to transport oxygen toward the retina.

Maintenance of a large oxygen pressure near the retina appears to be particular to teleosts and *Amia*. Data on the oxygen pressures in the eye of other vertebrates,

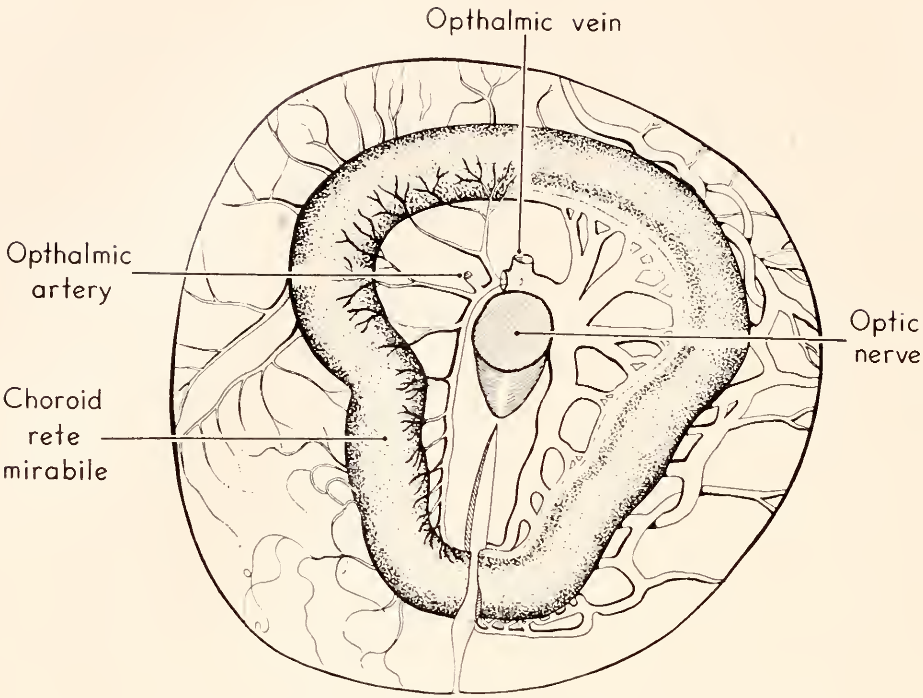

FIGURE 2. Choroid rete mirabile of the cod. Right eye viewed from inside the orbit. The right half shows the ophthalmic venous sinus and its drainage. On the left, the venous structures are dissected away to show the arteries.

collected in Table III, show only low pressures close to those expected of venous blood.

We now describe the anatomical relations of the teleost choroid rete mirabile. The choroid rete mirabile lies within the eyeball in the choroidal layer exterior to the retina and separated from the sclera by membranes which may, in different species, be laden with fat, or thin and unpigmented, or highly reflective and silvery, or intensely black. Silvery or black membranes may lie between the rete and the choriocapillaris (Denton, Liddicoat and Taylor, 1970). The rete itself is not pigmented. It has the shape of a horseshoe, with the open end oriented ventrally (Fig. 2) or, particularly in species in which the rete is large, anteriorly (Fig. 3). In species such as the bluefish in which the rete is large, the eye may lose its simple oblate shape and the sclera may bulge conspicuously to accommodate the bulk of the rete. There is no strong attachment of the rete to structures other than blood vessels in the interior of the eye. Figure 2 portrays the disposition of the choroid rete mirabile within the eye of a codfish viewed from inside the orbit. In this view the counter-current capillaries and small vessels are arrayed nearly normal to the plane of the paper and are hidden from view by the overlying ophthalmic venous sinus.

The ophthalmic artery pierces the sclera posterior, dorsal and close to the optic nerve, and passes into the ophthalmic venous sinus that forms the inner border of the choroid rete. Therein it divides into two branches, each supplying a limb of the rete. These branches may ramify into subsidiary branches running toward the rete proper as in the cod (Fig. 2); or they may be closely applied to the base of the capillaries and there arborize into stubby twigs at right angles to the main branch, as in bluefish (Fig.3). In either case, the arterial capillaries of the rete mirabile proper arise abruptly and almost directly from the thick-walled arteries (Fig. 4). The arteries run submerged in the ophthalmic venous sinus (Figs. 3 and 4).

The arterial retial capillaries coalesce to form a bed of intermediate arterial vessels which in turn coalesce into the arterial vessels supplying the choriocapillaris. These intermediate vessels are by no means a negligible part of the structure. In Figure 4 we attempt to show them in true proportion; their aggregate volume must exceed considerably the aggregate volume of the capillary vessels of the rete proper. In some larger fishes, most conspicuously in the gempylid, *Lepidocybium flavobrunneum*, but also in many others, the rete proper is only a thin sheet capping an enormous, tangled but roughly parallel array of coarse intermediate vessels filling much of the back of the eye, suggesting a mass of spaghetti.

The efferent arteries break up into fine arteries in the vascular layer underlying the retina (Fig. 4), and these arteries in turn supply the capillaries of the choriocapillaris. Ordinarily these vessels are as small as they are portrayed in Figure

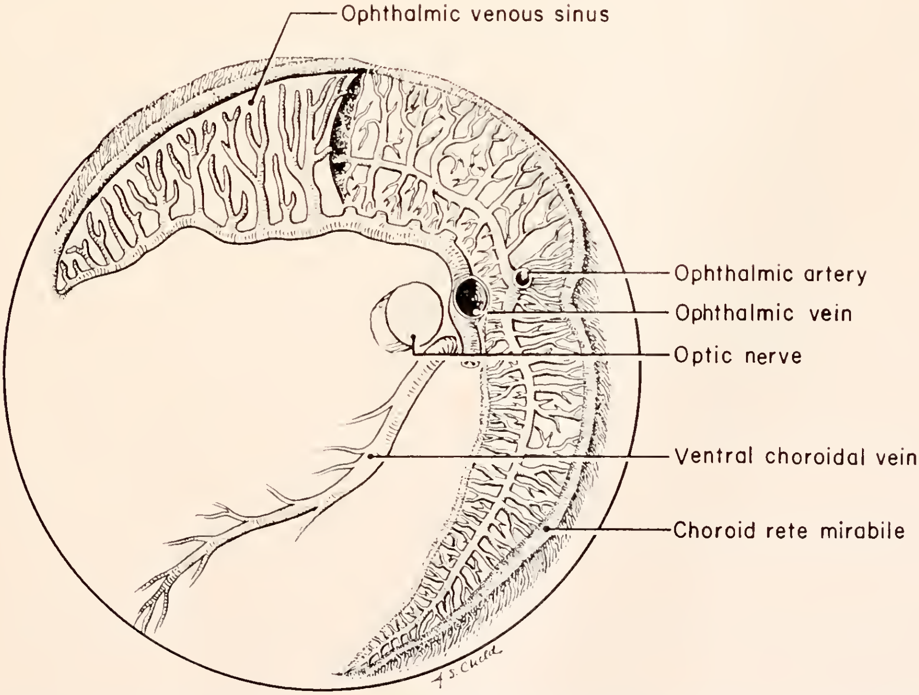

Figure 3. Choroid rete mirable of the bluefish. Right eye viewed from inside the orbit. The ophthalmic venous sinus is shown largely removed to display the underlying arteries.

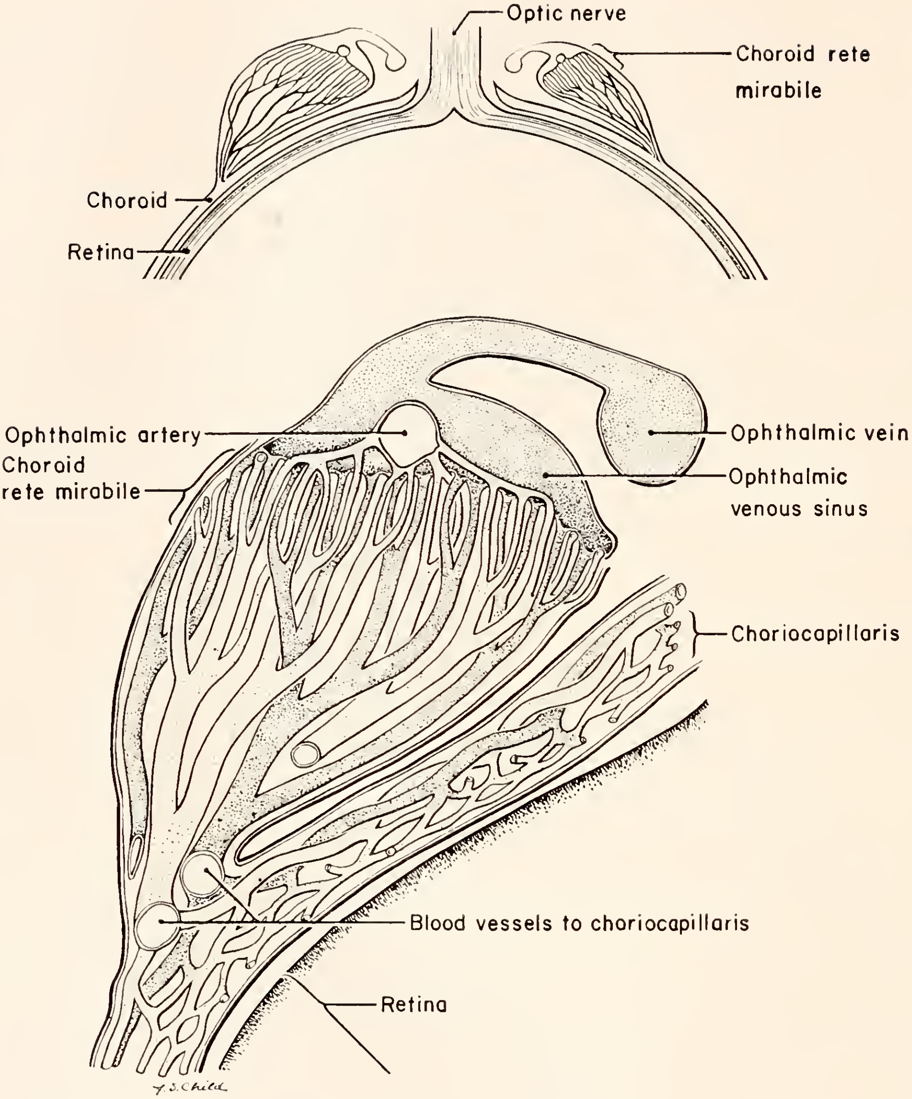
Optic nerve
Choroid rete mirabile
Choroid
Retina
Ophthalmic artery
Choroid rete mirabile
Ophthalmic vein
Ophthalmic venous sinus
Choriocapillaris
Blood vessels to choriocapillaris
Retina
J. S. Child

FIGURE 4. Semidiagrammatic representation of the choroid rete mirabile of the bluefish seen in longitudinal section. For clarity the size of the capillaries is exaggerated. The insert places the choroid rete in relation to the optic nerve and retina.

4. In the swordfish (*Xiphias gladius*), these vessels elaborate to form a hexagonal mesh of confluent vessels of rather large caliber (approximately 0.5 mm diameter) underlying the entire choriocapillaris and supplying it with blood by way of fine sprigs arising directly from the coarse vessels. The volume of blood contained in these vessels of the swordfish and in the intermediate arterial vessels noted in *Lepidocybium* must be large. Possibly it serves as a mobile reservoir of heat,

generated metabolically and conserved within the eye by the action of the choroid rete mirabile. In some species a carotid rete such as that described for the tuna by Linthicum and Carey (1972) may act in consort with the choroid rete as a barrier to heat loss. Some larger fish do maintain a high retinal temperature (Linthicum and Carey, 1972). A mobile reservoir of blood may help to dissipate the thermal gradients which Linthicum and Carey show to arise from unequal cooling of the eye. Consistent with this hypothesis is the fact that the vital structures of swordfish and gempylid eyes are blanketed by a thick layer of fat between the choroid and sclera.

The venous outflow from the choriocapillaris returns through small vessels to the venous capillaries of the rete mirabile proper. These empty without intervention of venules, directly into the ophthalmic venous sinus (Fig. 4). The ophthalmic vein drains the venous sinus and emerges through the sclera close to the optic nerve and separately from the ophthalmic artery.

TABLE IV

Dimensions of retia mirabilia

	Area supplied		Rete dimensions		Arterial capillaries		Venous capillaries	
	Choriocapillaris and retina	Gas gland	Capillary length	Cross section area	Number	Diameter	Number	Diameter
	cm^2	cm^2	cm	cm^2	millions	micrometers	millions	micrometers
Choroid rete								
Tuna	31		0.18	8.4	7.94	10.5	8.14	23.2
Swordfish	44		0.22	5.6	7.21	15	3.88	18
Average of 86 teleosts			0.14					
Swimbladder rete								
Eel		43	0.77	0.86	0.67	21.6	0.356	33.7
Conger		30	0.49	0.26	0.13	25.2	0.066	51.0
Bluefish			0.36					

The rete mirabile proper consists of many thousands of closely arrayed and parallel arterial and venous capillaries in which the afferent and efferent blood streams flow counter-current one to another. Partially injected preparations of the choroid rete mirabile of the bluefish, in which only arterial vessels contain the injection mass, identify the thick-walled capillaries of the rete as arterial and thin-walled capillaries as venous. Close-packing of the capillaries to achieve optimal exchange of materials is demonstrated by their orderly array seen in cross-section.

The capillaries may be arrayed as in a checkerboard, for instance in the tuna eye, in which case each venous capillary is surrounded by four arterial capillaries and the numbers of arterial and venous vessels are equal (Table IV). Or, as in the swordfish eye, the capillaries may be in hexagonal array, in which case each venous capillary is surrounded by six arterial capillaries and the number of arterial vessels is twice the number of venous vessels (Table IV). These arrangements of capillaries are illustrated diagrammatically in Figure 5. Less regular arrays in which each capillary tends to be surrounded by five neighbors are also en-

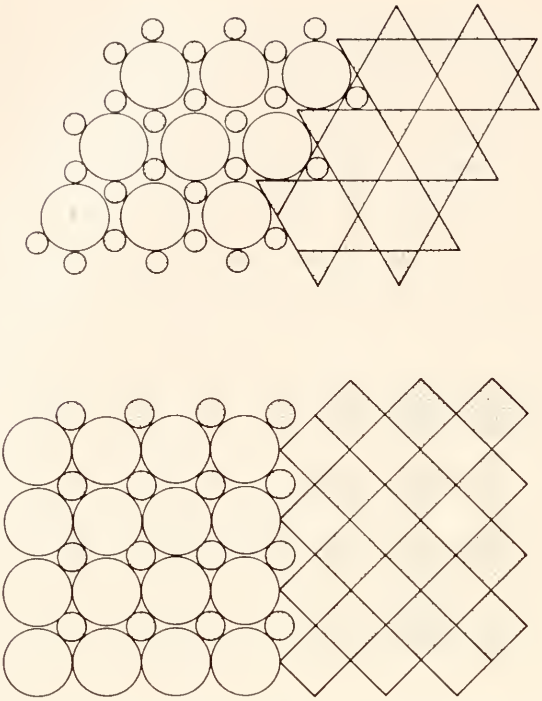

FIGURE 5. Diagram showing two ways in which arterial capillaries (stippled) and venous capillaries (white) may be arranged in a rete mirabile.

countered. We have not done serial sections, and cannot say whether these packing-patterns persist unchanged along the length of the rete.

An important aspect of retial structure, which must be taken into account in formulating any theory of the action of the counter-current system is that the choroid rete is not uniform along the length of the capillaries. In longitudinal sections of the rete of all fish, but particularly conspicuous in the rete of larger fish such as tuna or opah (lampridae, *Lampris regius*), the rete is seen clearly to be differentiated into proximal and distal portions of differing structure, with a sharp line of demarcation between them. This suggests that oxygen transfer by the choroid rete may be a two-stage process. The way in which the structures of the two parts of the rete differ is not easily resolved at the light microscope level. A study of the fine structure of the retial capillaries has been initiated in the laboratory of Copeland (*e.g.,* Wolley and Copeland, 1970) and we await their resolution of this problem.

We next describe the choroid rete mirabile of *Amia.* The appearance of this rete, viewed from inside the orbit, is portrayed in Figure 6. The sclera, covering membranes and the ophthalmic venous sinus have been dissected away. As in teleosts, the ophthalmic artery divides into two main branches, which arborize over the surface of the rete. The elegant and ordered symmetry of the teleost rete is lacking, and, in contrast to the teleost pattern, the retial capillaries arise from stubby branches of the larger arteries (Fig. 6). Aside from these minor differences, we find no essential difference between the choroid retia mirabilia of *Amia* and of teleosts.

D. W. Fawcett, Harvard Medical School (personal communication) has made electron micrographs of the choroid rete of *Amia.* His drawings, based on these micrographs (Fig. 7), contrast the structure of the capillaries of the choroid rete of *Amia* with those of the swimbladder rete of the toadfish, *Opsanus tau.* (Fänge and Wittenberg, 1958, describe the swimbladder of the toadfish.) He finds the choroid rete to differ from that of the toadfish swimbladder rete in several respects. (1) The venous channels are not discrete, straight vessels parallel to the arterial capillaries, but appear to form a labyrinthine system of sinusoidal vessels which often seem to surround the arterial capillaries. Wolley and Copeland (1970) find that venous channels in the teleost rete may also coalesce. (2) The differences in thickness of the walls of the two classes of vessels is very slight in striking contrast to the swimbladder rete. (3) The endothelial cell junctions which are simple in the swimbladder and show prominent desmosomes, are more imbricated and inter-

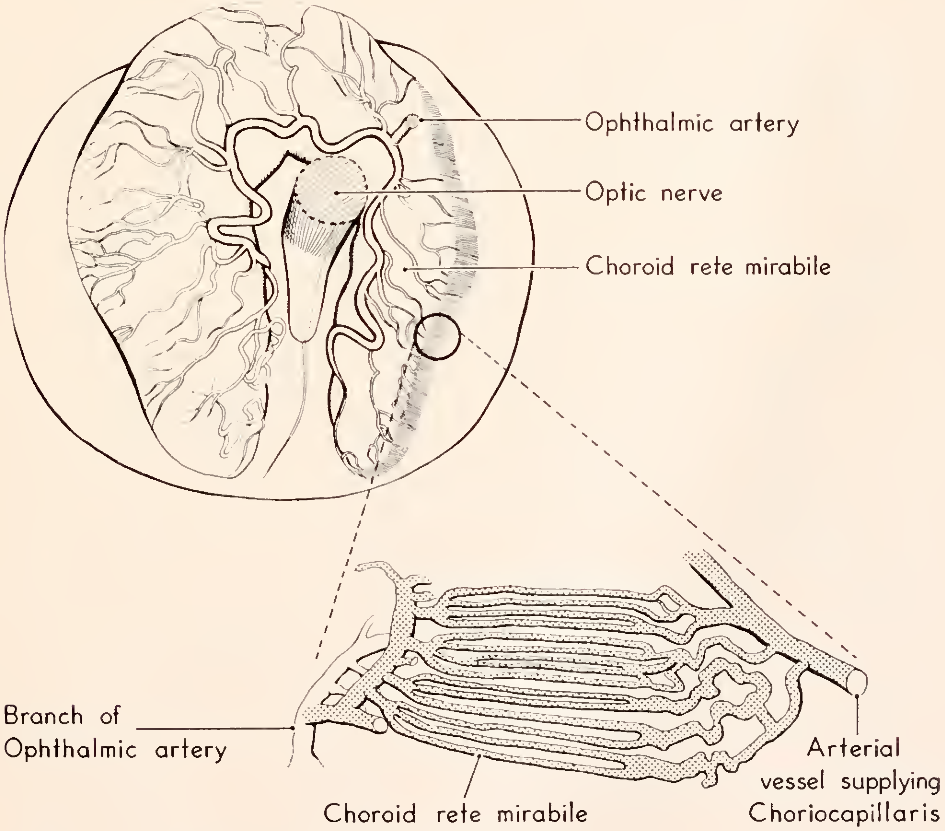

FIGURE 6. Choroid rete mirabile of the eye of *Amia.* Right eye viewed from inside the orbit. Only arterial structures are shown. The insert shows the arterial capillaries of the choroid rete at greater magnification.

digitated in the choroid rete and have no desmosomes. (In this respect they resemble mammalian capillaries more than do the toadfish capillaries.) (4) A particulate component of the plasma is rather consistently about five-fold more concentrated in the venous than in the arterial vessels.

It is of interest to compare the choroid and swimbladder retia mirabilia. The general features of the choroid rete of teleosts and of *Amia* are similar to those of the swimbladder rete mirabile but differences are noted: (1) The composition of the blood reaching the two retia may differ (Wittenberg and Haedrich, 1974) since blood comes to the choroid rete via the pseudobranch and to the swimbladder

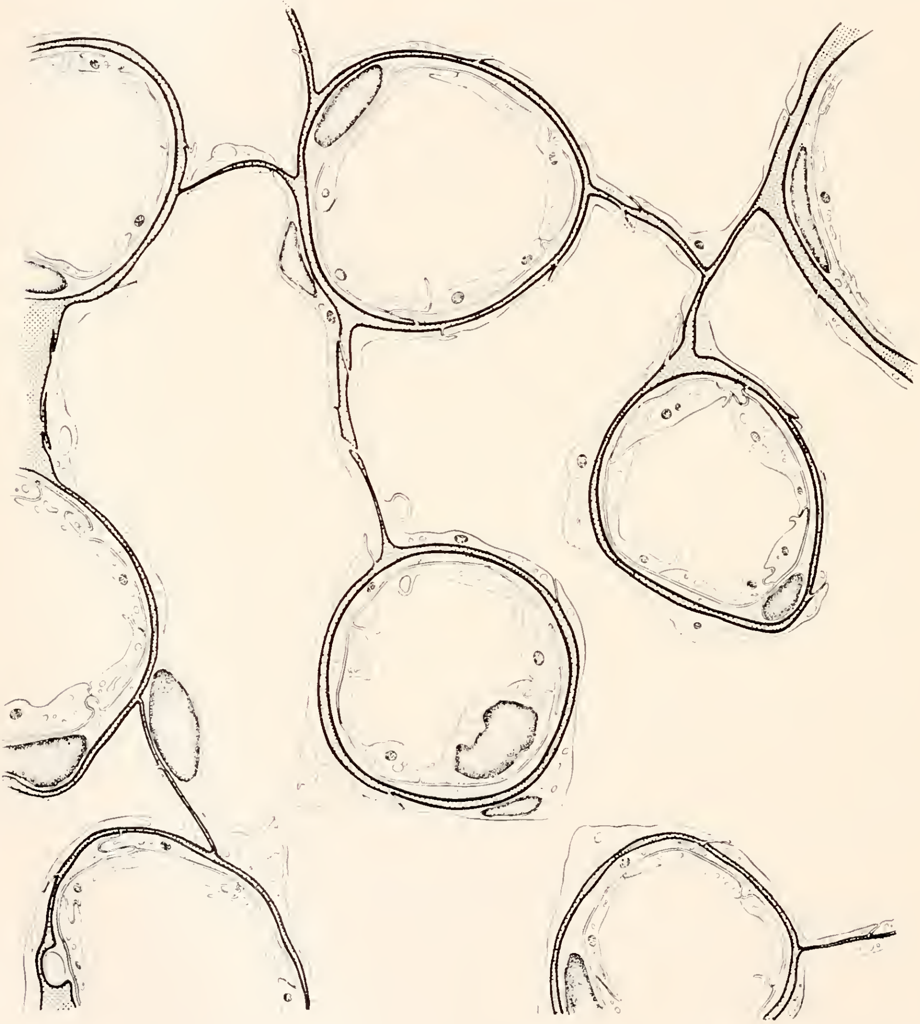

FIGURE 7. Diagrammatic cross-sections of the choroid rete of *Amia*, above, and the swimbladder rete of the toadfish, opposite; drawn from electron micrographs by Dr. Don W. Fawcett.

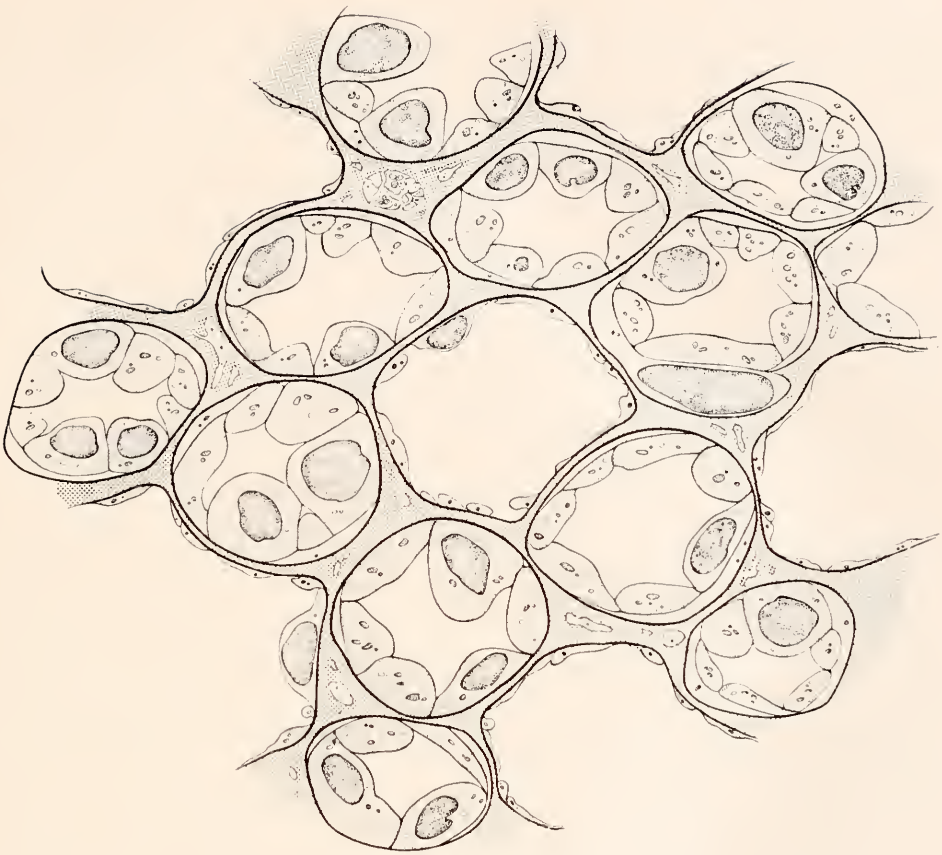

Figure 7. (*Continued*)

rete from the general circulation. (2) The difference in thickness of the walls of arterial and venous capillaries is very slight in the choroid rete of *Amia*. In contrast the arterial capillaries of the swimbladder rete are distinguished from those of the venous capillaries in that they are exceptionally thick and their cytoplasm contains a great abundance of smooth-surfaced vesicles (Fawcett and Wittenberg, 1959; Dorn, 1961; Fahlén, 1967; Jasiński and Kilarski, 1971). This points to some intracellular process more developed in the swimbladder than in the choroid rete. (3) The capillaries of the choroid rete are sharply differentiated into proximal and distal portions of differing structure; the capillaries of the swimbladder rete appear the same throughout. Although it is reasonable to consider that the general pattern of the physico-chemical mechanism by which large oxygen pressures are established is the same in the choroid and swimbladder retia, the structural differences are sufficient to suggest substantial differences in detail.

Krogh, in his celebrated book *The Anatomy and Physiology of the Capillaries* (1922), reports quantitative measurements of the capillaries of the rete mirabile of the swimbladder of an eel, and makes a plea that others should take up the study of quantitative anatomy. We begin by estimating the distance that oxygen must diffuse to supply the retina. If in fact the function of the system comprised by the choroid rete mirabile, the choriocapillaris and the pigment cell layer of the retina is to supply the retina with oxygen, we may enquire whether there is any unusual impediment to be overcome in the flow of oxygen from the capillaries of the choriocapillaris to the sites of oxygen consumption. The mitochondria of the photoreceptor cells are for the most part aggregated into a special structure, the spheroid, lying between the photoreceptive rod and cone outer segments and the respective cell soma. The distance from the inner surface of the choriocapillaris to the spheroid was measured in 26 representative species including carp, trout and 24 species of marine teleosts. The average distance from the inner surface of the choriocapillaris to the spheroid is 106 micrometers (range 78 to 170 micrometers). Although many teleosts have blood vessels (the retinal vessels) displayed on the inner surface of the retina, many do not, and all of the cells of the retina of these latter must depend on the choriocapillaris for their supply of oxygen. The average retinal thickness for the same sample was 360 micrometers (range 240 to 550 micrometers). These distances, across which diffusion must occur in the fish retina, are large. Compare for instance red muscle in which the diffusion path, one half the intercapillary distance, does not exceed 20 micrometers (Wittenberg, 1970). On the other hand, the distance which oxygen must diffuse in the primate retina is larger than in many other tissues. The retinal circulation of the primate penetrates from the inner face of the retina only as far as the outer plexiform layer; the layers corresponding to the photoreceptors are avascular and must depend for their oxygen on diffusion from the retinal and choroidal circulations (Polyak, 1957); the diffusion path may be of the order of 60 micrometers (Polyak, 1941). We note that the diffusion path in the fish retina is very long indeed and exceeds by more than six fold the diffusion path in the primate retina.

In Table IV we compare the dimensions of two swimbladder retia and two choroid retia supplying blood to capillary beds of about the same area. The task of the swimbladder rete is to maintain a volume of gas in the lumen in the face of the ambient pressure. The capillaries are of about the same length in two shallow-living species, bluefish and conger, whose patterns of gas secretion are quite different (Wittenberg, Schwend and Wittenberg, 1964; Fänge and Wittenberg, 1958). Marshall (1960), who has studied the swimbladders of deep sea fishes, notes that the length of the capillaries of the swimbladder rete increases with the depth at which the fish lives; the capillaries in abyssal fish may be 2.5 cm long; while the number of capillaries is proportional to the size of the fish. Following Marshall, we consider the eel, Table IV, as an example of a deep sea fish, albeit captured from shallow water. The retial capillaries are long, but the number of capillaries, and by that token, the cross-section area of the rete are not great.

The teleost retina consumes oxygen vigorously (Hoffert and Fromm, 1972), and the question arises how the choroid rete is adapted to meet this demand. A measure of the demand is given by the data of Hoffert (private communication) who finds that the retina of trout eyes (fish weight 98 gram, 15° C) consume 40.8 μliter O_2 per hour per eye, which is equivalent to 9.02 μliter O_2 per hour per cm^2 retinal area. From the data in Table IV we estimate that each cm^2 of retina is served by approximately 16–25 million arterial capillaries of the choroid rete.

The capillaries of the choroid rete are relatively short; the average length in teleosts is 0.14 cm (range of 86 teleosts, 0.052 to 0.18 cm; standard deviation 0.42). Although the fish in our sample weighed from a few grams to over 200 kilograms, the length of the capillaries was remarkably similar in all, and capillary length showed no correlation with body size. The average length of the capillaries of the rete of *Amia* was 0.21 cm. Dissection of the eyes of many species reveals that the increased demand accompanying larger size is met by a proliferation of a large number of short capillaries, so that the choroid rete mirabile in very large fish becomes a veritable sheet of tissue with capillaries arrayed normal to the plane of the sheet. This is exemplified by data on the tuna eye, Table IV, in which the rete contains 7.94 million arterial and 8.14 million venous capillaries, sixteen million in all, arrayed in parallel and each only 0.18 cm long. Their aggregate length is 2900 meters, and, as Krogh (1922) points out, this arrangement allows a very large surface for the exchange of substances to be provided within a small volume.

We conclude that the choroid rete mirabile is adapted to deliver a large flow of oxygen to the retina at the relatively low pressure required to overcome the barrier imposed by the long diffusion path in the retina.

We turn to a discussion of the role which movements of water may play in the physiology of the choroid and swimbladder retia. Electron micrographs of the choroid rete of *Amia calva*, mentioned above, and also of the swimbladder rete of *Opsanus tau*, reveal significant differences in the concentration of the plasma in the lumen of the arterial and venous capillaries, suggesting that there may be a considerable flux of water from one set of capillaries to the other. The direction of these fluxes is not established by the electron micrographs because we do not know from where in the rete the samples were taken. We now enquire whether substantial movements of water between the two classes of vessels of the rete mirabile may not be a part of the normal operation of the counter-current system secreting oxygen, in this instance into the swimbladder of the eel, *Anguilla vulgaris*.

Data bearing on this point are available from the elegant experiments of Steen (1963), who analyzed blood drawn from each of the blood vessels of the gas-secreting complex of the eel swimbladder during states of sustained oxygen secretion. The vessels from which blood was drawn are indicated as A, B, C and D in Figure 1. We focus attention on lactic acid. During gas secretion, aerobic glycolysis by the cells of the gas gland generates lactic acid which enters the blood flowing from points B to C. This is at once apparent from inspection of Steen's data, since the concentration of lactic acid is, in every experiment, much greater in blood leaving the gland at C than in blood entering at B. Steen, in interpreting his data, tacitly assumes that water neither enters nor leaves the capillaries of the rete. On that basis material balance of lactic acid is not achieved. The difference in concentration between the inflowing and outflowing blood (C_D-C_A) does not equal the difference across the gas gland, nor is the apparent loss of lactic acid in the venous capillary matched by an equivalent gain in the arterial vessels. This is not satisfactory. On the other hand, an internally consistent interpretation of the data emerges if we assume that: (1) Water may move between the two classes of capillary in the rete. (2) The sustained state of oxygen secretion studied by Steen was in fact a steady state. And, (3) lactic acid is not destroyed in the rete. The magnitude and direction of the water fluxes may be deduced.

All calculations are per unit time. Consider a unit volume of blood, V, entering the complex at A and containing X millimoles of lactic acid. The concentration of lactic acid at A, C_A, will be:

$$C_A = \frac{X}{V} \tag{1}$$

Consider that an amount of lactic acid, Y, passes between the arterial and venous capillaries. Considered also that a volume of water, ΔV, moves between the arterial and venous capillaries. The concentration of lactic acid at B, C_B, will be:

$$C_B = \frac{X - Y}{V - \Delta V} \tag{2}$$

There is no opportunity for water exchange in the capillaries of the gas gland. We note that an amount of lactic acid, Z, is added to the blood by glycolysis occurring in the cells of the gas gland. The concentration of lactic acid at point C, C_C, therefore will be:

$$C_C = \frac{X - Y + Z}{V - \Delta V} \tag{3}$$

Finally, since at steady state the volume of fluid leaving is equal to the volume entering, we have for the concentration of lactic acid at D, C_D:

$$C_D = \frac{X + Z}{V} \tag{4}$$

At steady state the amount of lactic acid added to the blood by the cells of the gas gland, Z, is equal to the added lactic acid, Z, leaving the rete at point D. Combining expressions 1 and 4, and 2 and 3, we have two expressions in Z, which do not involve Y. Combining these, we express ΔV in terms of V and of the experimentally measured lactic acid concentrations:

$$\frac{\Delta V}{V} = 1 - \left(\frac{C_D - C_A}{C_C - C_B}\right) \tag{5}$$

Here $\Delta V/V$ is the fraction of the total blood flow which passes from arterial to venous capillaries.

We obtain two expressions for Y/V, the amount of lactic acid moving between the arterial and venous capillaries per unit blood flow, as a function of $\Delta V/V$ and of the lactic acid concentrations at points A and B or at points C and D.

$$\left(\frac{Y}{V}\right)_{A,B} = C_A + C_B\left(\frac{\Delta V}{V} - 1\right) \tag{6}$$

$$\left(\frac{Y}{V}\right)_{C,D} = C_D + C_C\left(\frac{\Delta V}{V} - 1\right) \tag{7}$$

Steen's data for the lactic acid concentrations in the vessels of the gas secreting complex, together with the derived values $\Delta V/V$, $(Y/V)_{A,B}$ and $(Y/V)_{C,D}$ are presented in Table V. The agreement in the values of $(Y/V)_{A,B}$ and $(Y/V)_{C,D}$, respectively lactic acid leaving the arterial and entering the venous capillaries,

TABLE V

Calculated movement of water from arterial to venous capillaries of the eel swimbladder rete mirabile. The concentrations of lactic acid in the blood drawn from the retial vessels are those determined by Steen (1963). Blood vessels A, B, C, D are indicated in Figure 1.

Experiment	Lactic acid concentration in blood of vessels A, B, C, D				Derived values		
	C_A	C_B	C_C	C_D	$\left(\frac{Y}{V}\right)_{A,B}$	$\left(\frac{Y}{V}\right)_{C,D}$	$\frac{\Delta V}{V}$
	mM	mM	mM	mM	mM	mM	
A	2.7	3.7	7.8	3.4	2.1	2.1	0.83
B	9.8	14.9	20.9	13.1	1.6	1.6	0.45
C	3.2	4.4	10.9	5.1	1.9	1.9	0.71
D	4.4	5.7	11.1	5.9	2.8	2.8	0.72
E	6.2	9.1	16.9	9.9	1.9	1.9	0.53
F	8.8	8.9	12.3	8.9			
G	1.6	5.0	10.2	6.7	−3.4	−3.4	0.02
H	3.5	8.9	16.1	8.0	−2.0	−2.0	0.38

shows that calculations based on the assumption of water movement are internally consistent.

The value of $\Delta V/V$ is large in six of Steen's eight experiments, showing a net water flow from arterial to venous capillaries of the rete. Actually, in light of the fact that the hematocrit in these experiments ranged from 37 to 50 per cent, the calculated value of ΔV is improbably large, no doubt reflecting a systematic error.

The direction of movement of lactic acid is of some interest. A flux of lactic acid from a region of high concentration, the venous capillaries, to a region of lower concentration, the arterial capillaries, might be expected if the capillary walls are permeable to lactic acid. In Steen's experiment G the water flow is negligible, in his experiment H it is small; in these two experiments we note a flow of lactic acid down the concentration gradient. In each of his other experiments lactic acid apparently accompanies the moving water from arterial to venous capillaries.

Kuhn and his collaborators have proposed that oxygen concentration by the swimbladder rete is achieved by means of a counter-current multiplication mechanism (*e.g.*, Kuhn, Ramel, Kuhn and Marti, 1963; for a simple statement of Kuhn's hypothesis see Wittenberg, Schwend and Wittenberg, 1964). The operation of this mechanism requires that the lactic acid concentration in the outflowing venous vessels of the rete exceed that in the arterial vessels. Steen's data demonstrate that this requirement is in fact met in the working rete. We ask whether the movement of water from arterial to venous vessels does not oppose the establishment of the required large concentration of lactic acid in the venous vessels. The calculated quantity, $\Delta V/V$, represents a net movement of water along the whole length of the retial capillaries. If the bulk of this water movement takes place near the distal (swimbladder) pole of the rete the crucial concentration of lactic acid in the venous capillaries would be much dissipated. If, on the other hand, the bulk of the water movement occurs near the proximal (cardiac) pole of the rete, by increasing the concentration of solutes in the distal part of the rete, it might have the effect of enhancing the operation of the counter-current multiplication system.

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SUMMARY

Very large pressures of oxygen are found in the vitreous humor close to the retina of many teleosts and of the holostean, *Amia*. Elevated pressures are not found in species lacking a choroid rete mirabile. In species having choroid retia, the observed oxygen pressure roughly parallels the extent to which the rete is developed. On this evidence we suggest that the choroid rete mirabile, acting in concert with the pigment cell layer of the retina, plays an essential part in establishing the large oxygen pressure at the retina.

The choroid rete mirabile of the holostean, *Amia calva*, is essentially similar to the teleost choroid rete, from which it differs only in details of structure.

The capillaries of the counter-current organ, the choroid rete, are short, compared to the capillaries of the swimbladder rete, and are very nearly the same length in all the fishes examined, independent of the size of the fish, which ranged from tens of grams to hundreds of kilograms. The increased demand accompanying larger size is met by increasing the number of capillaries. We conclude that the structure of the choroid rete mirabile is adapted to supply a large flux of oxygen toward the retina at a relatively low pressure.

The general features of the structure of the choroid rete mirabile are largely similar to those of the swimbladder rete mirabile, but the two retia differ sufficiently to suggest that the physico-chemical mechanisms by which they build up large oxygen pressures may also differ in detail.

It is suggested that movements of water between inflowing and outflowing capillaries of the rete mirabile may play an important role in the mechanism by which oxygen is secreted into the eye and into the swimbladder.

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THE CHOROID RETE MIRABILE OF THE FISH EYE. II. DISTRIBUTION AND RELATION TO THE PSEUDOBRANCH AND TO THE SWIMBLADDER RETE MIRABILE.[1]

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The choroid rete mirabile is a large discrete organ lying within the eyeball behind the retina of many fishes. It is made up of several thousand closely arrayed and parallel arterial and venous capillaries in which the afferent and efferent blood streams flow counter-current one to another. This organ supplies arterial blood to the choriocapillaris (the dense capillary network underlying the retina), and in turn receives the venous outflow from these capillaries (Barnett, 1951). Probably in conjunction with the pigment cell epithelium, the choroid rete acts to maintain a large pressure of oxygen at the retina (Wittenberg and Wittenberg, 1962, 1974).

This investigation was undertaken to determine whether the distribution of the choroid rete mirabile in fishes is correlated with habitat, and, in particular, whether it is to be found in deep-living fishes. We find the choroid rete in both shallow- and deep-living fishes, and conclude that the ability to do without a choroid rete typifies families or orders of teleosts. Within such families or orders, the entire group, particular subgroups or individual genera, may lack the choroid rete. Among non-teleosts, the rete is present only in *Amia;* this may represent either an independent or a convergent evolutionary development. Within certain broad teleostean groupings, presence or, more particularly, absence of the choroid rete may indicate phylogenetic relationships. Two diverse, unrelated teleostean groups in which the choroid rete is absent are the eels (Anguilloidei) and the wholly deep-sea suborder Stomiatoidei. In the Scopelomorpha, the choroid rete is present in all families except the Myctophidae, emphasizing a dichotomy within the suborder recently predicated on other characters by Rosen and Patterson (1969). For the most part, however, presence or absence of the rete as a character should be used by evolutionists with the greatest care, and then only in a supporting role. We find it too widely spread, and perhaps too easily lost, to be of much use in unscrambling the complicated and intricate phylogeny of fishes.

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TABLE 1

Cyclostomes and elasmobranchs examined, none have a choroid rete mirabile.

Agnatha	
	Myxiniformes
Hagfish	*Myxine glutinosa**
	Petromyzontiformes
Sea lamprey	*Petromyzon marinus***
Brook lamprey	*Lampetra fluviatilis****
Chondrichthyes	
	Squaliformes
Bigeye thresher shark	*Alopias superciliosus*
White shark	*Carcharodon carcharias*
Smooth dogfish	*Mustelus canis*
Spiny dogfish	*Squalus acanthias*
Torpedo ray	*Torpedo nobiliana*
Winter skate	*Raja ocellata*
Sting ray	*Dasyatis centroura*
	Chaemaeriformes
Chimaera	*Hydrolagus colliei*

* Not examined. The eye is very much reduced; there is no choroid rete mirabile (Ross, 1963).

** Eyes and pineal eye examined.

*** N. A. Locket, Institute of Ophthalmology, London, private communication.

The pseudobranch, a modified gill, sits astride the blood supply to the eye, and arterial blood destined for the choroid rete must pass first through its capillaries. The oxygen-concentrating mechanism of any rete mirabile inevitably must also concentrate all diffusible substances for which there is a "primary concentrating effect." These include carbon dioxide present in the incoming blood. We advance the suggestion that the pseudobranch acts to modify the incoming arterial blood in such a way that the counter-current multiplication system of the choroid rete may concentrate oxygen without simultaneously building up an untoward concentration of carbon dioxide within the eye. Carbon dioxide when hydrated becomes a strong acid. The pattern of occurrence of the pseudobranch among salt, brackish, and fresh-water fishes provides a clue as to how this major function of the pseudobranch is accomplished.

MATERIALS AND METHODS

Eyes from specimens in museum or other collections were fixed for the most part in neutral formaldehyde and stored in 40 per cent isopropanol or 70 per cent ethanol. Eyes from fresh specimens were fixed in acid formalin (formalin, 10 volumes, glacial acetic acid, 5 volumes, water to 100 volumes) and were later transferred to 70 per cent ethanol. Histological sections were prepared from eyes transected in the saggital or horizontal plane. When the eye was small, serial sections were made. Identification of the choroid rete mirabile in most instances rests on both its gross appearance in dissected specimens and on its characteristic microscopic structure as seen in section. In all cases reported, the identification was unequivocal; particular care was taken not to miss an inconspicuous structure when the rete appeared to be absent. Except as noted, all statements that the rete is present or absent rest on our original observations. In the early and extensive studies of Brauer (1902, 1908), the choroid rete is not discussed, but is shown in his figures where it is identified as a "blood vessel" ("Blutgefäss").

The pseudobranch when present is usually easily visible on the inner face of the opercle. In the Gadiformes, Atheriniformes and in occasional genera (*e.g., Esox, Echeneis, Coryphaena*) of other groups, the pseudobranch is not evident to casual inspection but is revealed by dissection. The identity of the pseudobranch in these fishes was confirmed by histological examination. In some fresh water groups (*e.g.,* Characidae) the pseudobranch is small and is found only with difficulty. For this reason, it is occasionally difficult to be absolutely certain that a particular species lacks the pseudobranch. No dubious cases are included in Table VI.

We follow the recent classification of teleosts of Greenwood, Rosen, Weitzman and Meyers (1966), with the modifications of Greenwood, Myers, Rosen and Weitzman (1967), of Rosen and Patterson (1969), of Rosen and Greenwood (1970), and of Greenwood and Rosen (1971). We follow the phyletic relations of the sarcopterygian, chondrostean and holostean groups as given by Nelson (1969a, 1969b). Common names of fishes are those of Bigelow and Schroeder (1953) and the American Fisheries Society (1960).

Results and Discussion

There is no choroid rete mirabile in the eye of hagfish, lampreys, nor in the eyes of sharks, skates, rays, and chimaeras (Table I).

There is no choroid rete mirabile in the eyes of the lobe-finned fishes *Polypterus, Calamoichthys, Latimeria,* and the lungfishes *Protopterus, Lepidosiren,* and *Neo-*

Table II

Bony fishes examined other than teleosts. Of those here, only Amia *has a choroid rete mirabile.*

Sarcopterygii	
	Brachiopterygii
Bichir	*Polypterus unatipinnis*
Reedfish	*Calamoichthys calabaricus*
	Coelacanthini
Coelacanth	*Latimeria chalumnae**
	Dipnoi
African lungfish	*Protopterus aethiopicus*
South american lungfish	*Lepidosiren paradoxa*
Australian lungfish	*Neoceratodus forsteri*
Actinopterygii	
	Chondrostei
Sturgeon	*Acipenser oxyrhynchus*
Paddlenosed sturgeon	*Scaphirhynchus platyrhynchus*
Paddlefish	*Polyodon spathula*
	Holostei
Gar	*Lepisosteus osseus*
Bowfin	*Amia calva*

* See text.

ceratodus (Table II). Munk (1964, 1968b, 1969b) describes the structure of the eyes of some of these and compares them with *Amia, Lepisosteus* and teleosts.

The eye of the coelacanth, *Latimeria chalumnae,* was not examined in this study. The eye has been described briefly by Millot and Carasso (1955) and by Millot and Anthony (1958a). Millot and Anthony (1958b, plates VI and XIV; 1965, plates LVIII and LX) present photographs of the eye transected in planes which would be expected to include the rete were it present; there is none visible. Dr. N. A. Locket, Institute of Opthalmology, University of London (private communication) has examined a coelacanth eye which had been divided sagittally; there is no suggestion of a rete, and histological sections confirm this observation.

There is no choroid rete mirabile in the eyes of the chondrosteans, sturgeons and the paddlefish (Table II).

There are two living holosteans, the gar and the bowfin. Of these, the gar, *Lepisosteus,* has no choroid rete. The bowfin, *Amia calva,* however, does, and is the only non-teleost fish we have found to have one (Table II). Wittenberg and Wittenberg (1974) describe the architecture of this rete. The structure of the holostean eye is described most recently by Munk (1968b). The rete of *Amia* may represent an independent evolutionary development convergent with that of the teleosts. On the other hand, it is equally possible that a common ancestor of *Amia* and of the teleosts may either have developed a choroid rete mirabile or had the genetic capability to develop one. Whether *Amia* and teleosts share an immediate common ancestor is at present a matter of debate; see a discussion by Nelson (1969a, 1969b). However this question is resolved, the choroid rete of *Amia* serves the same function as that of the teleosts—to maintain a large pressure of oxygen at the retina (Wittenberg and Wittenberg, 1974).

The choroid rete mirabile is widely distributed among teleosts (Table III). It is nearly ubiquitous among the Acanthopterygii which make up the great majority of living forms. It is lacking in eels (Anguilloidei) which are unique among fishes in having vascular retinas (Duke-Elder, 1958; Walls, 1942), possibly obviating the need for a choroid rete.

The Gadiformes present an instructive diversity. This group includes members, for example the cod, haddock, pollack, and silver hake, with powerfully-developed choroid retia. On the other hand, the rete is reduced to a minute, although certainly functional, structure in the three morids examined. These are relatively deep to very deep-living animals. The macrurids, typically deep-living forms, carry this tendency a step further; the rete of *Nezumia* is reduced in size almost to the vanishing point, while the other two species examined lack retia altogether. Two zoarcids were examined; the shallow-living *Macrozoarces* was found to have a normal-sized rete, while the deep-living *Lycodes,* captured from 1400 meters lacks a rete. One cannot, on this evidence, conclude that life at depth is the sole reason for loss of the rete—the two shallow-living ophidiids, *Lepophidium* and *Otophidium,* lack retia, as does their deep-living relative, *Dicrolene.* A more conservative conclusion is that the Gadiformes have the potential to do without the choroid rete. When it has selective advantage, the rete is powerfully developed, but it becomes reduced in species subject to selective pressures favoring its loss.

The loss of, or the ability to lose, the choroid rete is a character typifying certain families or larger groupings. Contrast, for instance, the stomiatoids, nearly

TABLE III

Distribution of the choroid rete mirabile and pseudobranch in teleosts. Plus indicates organ present. Zero indicates organ absent. Specimens not examined are designated n.e.

DIVISION I (TAENIOPAEDIA)			Choroid Rete	Pseudobranch
	Superorder Elopomorpha			
	Order Elopiformes			
	Suborder Elopoidei			
Ladyfish	Elopidae	*Elops saurus*	+	+
		Elops affinis	n.e.	+
Tarpon	Megalopidae	*Megalops atlantica*	+	+
		Megalops cyprinoides	+	+
	Suborder Albuloidei			
Bonefish	Albulidae	*Albula vulpes*	+	+
		Pterothrissus gissu	n.e.	+
		Dixonia nemoptera	+	+
	Order Anguilliformes			
	Suborder Anguilloidei			
American eel	Anguillidae	*Anguilla rostrata*	0	n.e.
Conger	Congridae	*Conger oceanicus*	0	n.e.
Moray	Muraenidae	*Muraena* sp.	0	n.e.
Snipe eel	Nemichthyidae	*Nemichthys scolopaceus*	0	n.e.
Longnose eel	Synaphobranchidae	*Synaphobranchus pinnatus*	0	n.e.
	Order Notacanthiformes[a]			
	Halosauridae	*Aldrovandia phalacra*	0	0
Spiny eel	Notacanthidae	*Notacanthus* sp.	0	0
		Polyacanthonotus rissoanus	0	0
	Superorder Clupeomorpha			
	Order Clupeiformes			
	Suborder Clupeoidei			
Anchovy	Engraulidae	*Anchoa mitchilli*	+	+
Hickory shad	Clupeidae	*Alosa mediocris*	+	+
Alewife		*Alosa pseudoharengus*	+	+
Menhaden		*Brevoortia gunteri*	+	+
Menhaden		*Brevoortia tyrannus*	+	+
Herring		*Clupea harengus*	+	+
DIVISION II (ARCHAEOPHYLACES)				
	Superorder Osteoglossomorpha			
	Order Osteoglossiformes			
	Suborder Osteoglosoidei			
Aruana	Osteoglossidae	*Osteoglossum bicirrhosum*	+	0
		Heterotis niloticus	+	0
		Scleropages guentheri	+	0
Arapaima		*Arapaima gigas*	+	0
		Scleropages formosus	+	n.e.
Butterfly fish	Pantodontidae	*Pantodon buchholzi*	+	0
	Suborder Notopteroidei			
Goldeye	Hiodontidae	*Hiodon alosoides*	+	0
Featherback	Notopteridae	*Notopterus notopteurs*	+	0
Featherback		*Notopterus chitala*	+	0
African knife fish		*Xenomystus nigri*	+	0
	Order Mormyriformes			
	Mormyridae	*Gnathonemus tomandua*	0	n.e.
		Gnathonemus petersii	0	0
		Gnathpnemus niger	0	0
	Gymarchidae	*Gymnarchus niloticus*	0	0
DIVISION III (EUTELEOSTEI)				
	Superorder Protacanthopterygii			
	Order Salmoniformes			
	Suborder Salmonoidei			
Whitefish	Salmonidae	*Coregonus clupeaformis*	+	+
Brown trout		*Salmo trutta*	+	+
Grayling		*Thymallus thymallus*	+	n.e.
Smelt	Osmeridae	*Osmerus mordax*	+	+
Capelin		*Mallotus villosus*	+	n.e.

TABLE III—*Continued*

DIVISION III (EUTELEOSTEI)—*Continued*			Choroid Rete	Pseudo-branch
	Suborder Argentinoidei			
Argentine	Argentinidae	*Argentina silus*	+	n.e.
Argentine		*Argentina sphyraena*	+	n.e.
		Nansenia groenlandica[b]	+	n.e.
	Bathylagidae	*Bathylagus pacificus*[b]	+	n.e.
		Bathylagus stilbius[b]	+	n.e.
		Bathylagus longirostris	+	+
		Bathylychnops exilis[b]	+	n.e.
		Opisthoproctus grimaldii[b]	+	n.e.
		Rhynchohyalus natalensis[c]	+	n.e.
		Winteria telescopa[b]	+	+
	Alepocephalidae			
		Alepocephalus agassizi	+	+
		Platytroctegen mirus[b]	+	n.e.
		Bathylaco nigricans[d]	+	+
	Suborder Stomiatoidei			
	Gonostomatidae	*Gonostoma elongatum*[b,e]	0	0
		Vinciguerria poweriae	0	0
Hatchetfishes	Sternoptychidae	*Argyropelecus* sp.[e]	+	n.e.
		Argyropelecus aculeatus	n.e.	+
		Argyropelecus olfersi[b]	0	+
		Sternoptyx sp.[e]	+	+
	Astronesthidae	*Astronesthes indicus*[b]	0	n.e.
		Astronesthes niger	0	0
		Borostomias antarcticus	0	0
	Melanostomiatidae	*Bathophilus pawneei*[b]	0	n.e.
		Bathophilus metallicus[b]	0	0
		Eustomias obscurus[b]	0	0
		Flagellostomias boureei[b]	0	n.e.
		Melanostomias spilorhynchus[b]	0	0
	Malacosteidae	*Malacosteus niger*	0	0
Viperfishes	Chauliodontidae	*Chauliodus schmidti*	0	0
		Chauliodus sloani[b]	0	0
		Chauliodus sp.[e]	0	n.e.
	Stomiatidae	*Stomias* sp.[b]	0	n.e.
		Stomias boa	n.e.	0
	Idiacanthidae	*Idiacanthus fasicola*[b]	0	0
	Suborder Esocoidei			
Pickerel	Esocidae	*Esox niger*	+	0
Mud minnow	Umbridae	*Umbra limi*	+	0
	Superorder Ostariophysi			
	Series Anotophysi			
	Order Gonorynchiformes			
	Suborder Chanoidei			
Milkfish	Channidae	*Chanos chanos*	+	+
	Series Otophysi			
	Order Cypriniformes			
	Suborder Characoidei			
	Characidae	*Alestes kingsleyae*	+	+
		Brycon striatulus	+	+
		Creatochanes melanurus	+	+
Piranha		*Serrasalmus nattereri*	+	+
South American knifefish	Gymnotidae	*Eigenmannia virescens*	0	0
South American knifefish	Apteronotidae	*Sternarchus albifrons*	0	0
	Suborder Cyprinoidei			
Carp	Cyprinidae	*Cyprinis carpio*	+	n.e.
Goldfish		*Carassius auratus*	+	n.e.
Tench		*Tinca vulgaris*	+	+
Sucker	Catostomidae	*Catostomus commersoni*	+	+
	Order Siluriformes			
Catfish	Ictaluridae	*Ameriurus nebulosus*	0	n.e.
Catfish	Bagridae	*Bagrus docmac*	0	n.e.
Gafftopsail catfish		*Bagrus marinus*	0	0
Walking catfish	Clariidae	*Clarias batrachus*[e]	0	n.e.
	Superorder Scopelomorpha			
	Order Myctophiformes			
Lizard fish	Synodontidae	*Synodus foetans*	+	+
		Synodus sp.	+	+
	Omosudidae	*Omosudis loweii*[b,f]	+	+
Lancetfish	Alepisauridae	*Alepisaurus brevirostris*	+	+
	Evermannellidae	*Evermannella indica*[b]	+	+

TABLE III—*Continued*

DIVISION III (EUTELEOSTEI)—*Continued*			Choroid Rete	Pseudobranch
	Scopelarchidae	*Neoscopelarchoides* sp.[b]	+	+
		Scopelarchus sp.[e]	+	+
Lanternfishes	Myctophidae	*Ceratoscopelus maderensis*	0	+
		Lampanyctus macdonaldi	0	+
		Myctophum punctatum	0	+
		Symbolophorus veranyi	0	+
	Neoscopelidae	*Scopelengys tristis*	0	0
	Superorder Paracanthopterygii			
	Series Polymixiomorpha			
	Order Polymixiiformes			
Beardfish	Polymixiidae	*Polymixia nobilis*	+	+
	Series Salmopercomorpha			
	Order Percopsiformes			
Troutperch	Percopsidae	*Percopsis omiscomaycus*	+	+
	Order Gadiformes			
	Suborder Gadoidei			
Blue hake	Moridae	*Antimora rostrata*	+	0
		Halargyreus johnsonii	+	0
		Mora moro	+	n.e.
Cod	Gadidae	*Gadus morhua*	+	+
Freshwater cod		*Lota lota*	+	n.e.
Haddock		*Melanogramus aeglefinus*	+	n.e.
Tomcod		*Microgadus tomcod*	+	n.e.
Pollack		*Pollachius virens*	+	+
Rockling		*Enchelyopus cimbrius*	+	+
Longfin hake		*Urophycis chesteri*	+	+
Squirrel hake		*Urophycis chuss*	+	+
Spotted hake		*Urophycis regius*	+	+
White hake		*Urophycis tenuis*	+	n.e.
Silver hake (whiting)	Merlucciidae	*Merluccius bilinearis*	+	+
	Suborder Macrouroidei			
Rattails	Macrouridae	*Nezumia bairdii*	+	+
		Macrurus sp.	0	n.e.
		Macrurus tenuicauda	0	n.e.
	Suborder Ophidioidei			
	Ophidiidae	*Dicrolene intronigra*	0	0
Cusk eel		*Lepophidium cervinum*	0	+
Cusk eel		*Otophidium welshi*	0	0
	Suborder Zoarcoidei			
Ocean pout	Zoarcidae	*Macrozoarces americanus*	+	+
Eel pout		*Lycodes atlanticus*	0	0
	Order Batrachoidiformes			
Toadfish	Batrachoididae	*Opsanus tau*	+	0
Batfish		*Ogcocephalus nasutus*	+	n.e.
Midshipman		*Porichthys notatus*	0	+
	Order Lophiiformes			
	Suborder Lophioidei			
Goosefish	Lophiidae	*Lophius piscatorius*	+	0
	Suborder Antennarioidei			
Sargassum fish	Antennariidae	*Histrio pictus*	+	n.e.
	Suborder Ceratioidei			
Deep sea anglerfish	Ceratiidae	*Ceratias holboelli*[b]	0	n.e.
		Cryptopsaras cousi[b]	0	n.e.
	Linophrynidae	*Linophryne arborifera*[b]	0	n.e.
	Superorder Acanthopterygii			
	Series Atherinomorpha			
	Order Atheriniformes			
	Suborder Exocoetoidei			
Flying fish	Exocoetidae	*Exocoetus* sp.	+	n.e.
Flying fish		*Oxyporhamphus micropterus*	+	+
Flying fish		*Danichthys rondeletti*	+	+
Halfbeak		*Hyporhamphus unifasciatus*	+	+
	Belonidae	*Petalichthus capensis*	+	+
Houndfish		*Strongylura* sp.	+	n.e.
Needlefish		*Tylosurus acus*	+	+
	Scomberesocidae			
Saury		*Somberesox saurus*	+	0
	Suborder Cyprinodontoidei			
	Cyprinodontidae			
Sheepshead minnow		*Cyprinodon variegatus*	+	+
Mummichog		*Fundulus heteroclitus*	+	+
Mummichog		*Fundulus majalis*	+	+
Platy		*Xiphophorus* sp.	+	+
		Aplocheilus lineatus[g]	+	+
		Epiplatys grahami[g]	+	+

TABLE III—*Continued*

	DIVISION (III EUTELEOSTEI)—*Continued*		Choroid Rete	Pseudo-branch
	Anablepidae			
Foureyes		*Anableps tetrophthalmus*[b]	+	n.e.
	Series Percomorpha			
	Order Lampridiformes			
Opah	Lampridae	*Lampris regius*	+	n.e.
	Suborder Stylephoroidei			
	Stylephoridae	*Stylephorus chordatus*[b]	+	n.e.
	Order Beryciformes			
	Suborder Stephanoberycoidei			
	Melamphaeidae	*Poromitra nigrofulvus*[e]	0	n.e.
		Scopelogadus beanii	0	0
	Suborder Berycoidei			
	Diretmidae	*Diretmus argenteus*[i]	+	n.e.
	Order Zeiformes			
American john dory	Zeidae	*Zenopsis ocellata*	+	+
	Order Gasterosteiformes			
	Suborder Gasterosteoidei			
Stickleback	Gasterosteidae	*Gasterosteus aculeatus*	+	+
	Suborder Aulostomoidei			
Cornet fish	Fistulariidae	*Fistularia tabacaria*	+	+
	Suborder Syngnathoidei			
Seahorse	Syngnathidae	*Hippocampus hudsonius*	+	n.e.
Pipefish		*Syngnathus louisianae*	+	n.e.
	Order Channiformes			
Snakehead	Channidae	*Ophicephalus micropeltes*	+	0
Snakehead		*Channa asiatica*	+	0
	Order Scorpaeniformes			
	Suborder Scorpaenoidei			
Blackbellied rosefish	Scorpaenidae	*Helicolenus dactylopterus*	+	+
Scorpion fish		*Scorpaena brasiliensis*	+	+
Rosefish		*Sebastes marinus*	+	+
Sea robin	Triglidae	*Prionotus carolinus*	+	+
	Suborder Cottoidei			
Arctic sculpin	Cottidae	*Cottunculus microps*	0	0
Sea raven		*Hemitrypterus americanus*	+	+
Longhorn sculpin		*Myoxocephalus octodecimspinosus*	+	+
Lumpfish	Cyclopteridae	*Cyclopterus lumpus*	+	+
	Order Perciformes			
	Suborder Percoidei			
Wreckfish	Percichthyidae	*Polyprion americanus*	+	+
Striped bass		*Morone saxatilis*	+	+
Sea bass	Serranidae	*Centropristes striatus*	+	+
Sand perch		*Diplectrum formosum*	+	+
Sunfish	Centrarchidae	*Lepomis auritus*	+	+
Crappie		*Pomoxis annularis*	+	+
Largemouth bass		*Micropterus salmoides*	+	+
Yellow perch	Percidae	*Perca flavescens*	+	+
Walleye		*Stizostedion vitreum*	+	+
	Branchiostegidae			
Tilefish		*Lopholatilus chamaeleonticeps*	+	n.e.
Bluefish	Pomatomidae	*Pomatomus saltatrix*	+	+
Remora	Echeneidae	*Echeneis naucrates*	+	+
Bigeye scad	Carangidae	*Selar crumenophthalmus*	+	+
Dolphin	Coryphaenidae	*Coryphaena equiselis*	+	0
Black pomfret	Apolectidae	*Apolectus niger*	+	+
Snapper	Lutjanidae	*Etelis marshi*[e]	+	n.e.
Scup, porgy	Sparidae	*Stenotomus versicolor*	+	+
Sand seatrout	Sciaenidae	*Cynoscium arenarius*	+	+
Salt water drum		*Micropogon undulatum*	+	+
Fresh water drum		*Aplodinotus grunniens*	+	+
Spotted seatrout		*Cynoscium nebulosus*	+	+
Minkfish		*Menticirrhus focaliger*	+	+
Kingfish		*Menticirrhus saxatilis*	+	n.e.
Spadefish	Ephippidae	*Chaetodipterus faber*	+	+
	Suborder Mugiloidei			
Mullet	Mugilidae	*Mugil cephalus*	+	+
	Suborder Sphyraenoidei			
Northern barracuda	Sphyraenidae	*Sphyraena borealis*	+	+
Barracuda		*Sphyraena* sp.	+	n.e.
	Suborder Labroidei			
Tautog	Labridae	*Tautoga onitis*	+	+
Cunner		*Tautogolabrus adspersus*	+	+

TABLE III—*Continued*

	DIVISION III (EUTELEOSTEI)—*Continued*		Choroid Rete	Pseudobranch
Redtail parrotfish	Scaridae	*Sparisoma chrysopterum*	+	+
	Suborder Trachinoidei			
	Chiasmodontidae	*Chiasmodon* sp.	+	+
	Suborder Notothenioidei			
	Nototheniidae	*Notothenia cornucola*	+	n.e.
		Harpagifer bispinus	0	n.e.
Icefishes	Chaenicthyidae	*Chaenocephalus aceratus*	0	n.e.
		Champsocephalus gunnari	0	n.e.
	Suborder Blenniodei			
Florida blenny	Blennidae	*Chasmodes sabburae*	+	+
Wolffish	Anarhichadidae	*Anarhichus lupus*	+	+
Foureyed blenny	Clinidae	*Dialommus fuscus*[j]	+	n.e.
Wrymouth	Stichaeidae	*Cryptacanthodes maculatus*	0	n.e.
Rock gunnel		*Pholis gunnellus*	+	+
Shanny		*Leptoclinus maculatus*	+	+
	Suborder Scombroidei			
	Gempylidae	*Thyrsites atun*	+	+
Oilfish		*Ruvettus pretiosus*	+	+
		Lepidocybium flavobrunneum	+	+
Bonito	Scombridae	*Sarda sarda*	+	+
Mackerel		*Scomber scombrus*	+	+
King mackerel		*Scomberomorus cavalla*	+	n.e.
Bluefin tuna		*Thunnus thynnus*	+	+
White marlin	Istiophoridae	*Makaira albida*	+	n.e.
Swordfish	Xiphiidae	*Xiphias gladius*	+	+
	Suborder Stromateoidei			
Barrel fish	Centrolophidae	*Hyperoglyphe bythites*	+	+
Butterfish	Stromateidae	*Peprilus triacanthus*	+	+
Harvest fish		*Peprilus alepidotus*	+	n.e.
	Ariommidae	*Ariomma regulus*	+	+
	Suborder Anabantoidei			
Climbing perch	Anabantidae	*Anabas oligolepis*	+	0
Gourami	Helostomatidae	*Helostoma temmincki*	+	0
	Order Pleuronectiformes			
	Suborder Pleuronectoidei			
Winter flounder	Pleuronectidae	*Pseudopleuronectes americanus*	+	+
Yellow tail flounder		*Limanda ferruginea*	+	+
Sand flounder	Bothidae	*Lophopsetta maculata*	+	+
Fluke		*Paralichthys dentatus*	+	+
	Order Tetraodontiformes			
	Suborder Balistoidei			
File fish	Balistidae	*Monacanthus hispidus*	+	+
	Suborder Tetraodontoidei			
Puffer	Tetraodontidae	*Sphaeroides maculata*	+	+
Cowfish		*Lactrophrys tricornis*	+	n.e.
Spiny boxfish	Diodontidae	*Diodon hystrix*	+	n.e.
Striped burrfish		*Chilomycterus schopfi*	+	n.e.
Ocean sunfish	Molidae	*Mola mola*	+	n.e.

Notes to Table III:

[a] McDowell (1973) finds that the pseudobranch is absent throughout this order. We confirm his finding for *Aldrovandia* and *Notacanthus*; we did not examine the pseudobranch of *Polyacanthonotus*.

[b] Rete or its absence noted by Munk (1966a).

[c] Rete noted by Bertelsen, Theisen and Munk (1965).

[d] Rete noted by Munk (1968a).

[e] Rete or its absence noted by N. A. Locket, private communication.

[f] Rete noted by Munk (1965).

[g] Rete noted by Munk (1970). Pseudobranch described by Poernomo (1967).

[h] Rete noted by Arruga, quoted in Duke-Elder (1958).

[j] Rete noted by Munk (1966b).

[i] Rete noted by Munk (1969a).

all of which lack the choroid rete, with the other suborders of Salmoniformes, nearly all of which have choroid retia. The Salmoniformes are for the most part small mesopelagic animals, presumably living somewhat similar lives. Another clear example is encountered in the Scopelomorpha, where six families have the rete, and a single family, the speciose Myctophidae, lacks the rete.

The Notothenioids deserve comment. The two chaenichthyids studied are examples of the famous icefishes described by Ruud (1965) as having neither red blood cells nor hemoglobin in their blood. Also absent are swimbladders (Ruud, private communication) and choroid retia. It cannot be said whether the loss of these structures is secondary to the loss of red blood cells. In an attempt to find out, the eyes of two related nototheniids were examined. At least one of these, *Notothenia,* is known to have red blood (Tyler, 1960) and has a choroid rete. Another, *Harpagifer,* lacks the choroid rete.

We discuss next the relation between the occurrence of retia in the swimbladder and the eye. The retia of the swimbladder and of the eye are similar both in structure and in one known function—to secrete oxygen. The question arises whether species which dispense with one or the other of these structures do so because of some aspect of their physiology or their habitat which makes the operation of a counter-current organ ineffective. Table IV lists some fish lacking the choroid rete but having well-developed swimbladder retia and oxygen secretion into the swimbladder. Table V lists some examples of the converse case, species with well-developed choroid retia but with the swimbladder rete poorly developed or seemingly absent. These fishes secrete oxygen into the swimbladder, but very slowly. From these observations, we conclude that the swimbladder rete and the choroid rete occur independently.

TABLE IV

Fish lacking choroid retia but having well-developed swimbladder retia or oxygen secretion into the swimbladder.

		Reference to Swimbladder
Eel	*Anguilla rostrata*	Fänge and Wittenberg, 1958
Conger	*Conger oceanicus*	Richard, 1895
Moray	*Muraena helena*	Schloesing and Richard, 1896
Longnose eel	*Synaphobranchus pinnatus*	Schloesing and Richard, 1896
Spiny eels	*Notacanthiformes*	McDowell, 1973
	Vinciguerria sp.	Kanwisher and Ebeling, 1957 Marshall, 1960
	Argyropelecus olfersi	Marshall, 1960
	Astronesthes niger	Marshall, 1960
	Ceratoscopelus maderensis	Backus *et.al*, 1968
	Lampanyctus sp.	Marshall, 1960
	Myctophum sp.	Kanwisher and Ebeling, 1957 Marshall, 1960
	Porichthys notatus	This work
	Dicrolene intronigra	This work
	Otophidium welshi	This work
	Macrurus sp.	This work
	Poromitra sp.	Kanwisher and Ebeling, 1957

We discuss next the relation of the pseudobranch to the choroid rete mirabile. The pseudobranch is widely distributed among fishes and seldom lost. Those instances in which it is lost, therefore, offer an occasion for deductions about its function.

The pseudobranch is a modified (first, spiracular) gill arch which receives oxygenated blood from the first efferent gill artery and in turn gives rise to an artery of which the ophthalmic artery, supplying the choroid rete, is a main branch

(Allen, 1905; Allis, 1900, 1908, 1912, and references therein; Barnett, 1951; Müller, 1839; Prince, 1956). (Goodrich, 1930, and Prince, 1956, present useful figures.) Blood, passing from the heart to the eye and back to the heart passes through five tandem sets of capillaries as follows: the gill capillaries, the pseudobranchial capillaries, and, within the eye, the afferent (arterial) capillaries of the choroid rete mirabile, the capillaries of the chorio-capillaris underlying the retina and lastly the efferent (venous) capillaries of the choroid rete.

In many teleost species, the pseudobranch retains the external appearance of a gill hemibranch, but is distinguished from a gill by its location on the inner face of the opercle, where it shares access to the respiratory current of water. In other species (*e.g.*, many gadoids), the filaments of the pseudobranch are to a greater or lesser extent fused, so that diffusion from blood to sea water must be substantially impeded, and respiratory exchange minimized. Such pseudobranchs are often covered by a flap or layer of epithelium. In still others, the respiratory filaments are fused completely and the pseudobranch is a compact structure buried in connective tissue beneath the buccal epithelium. Among teleosts both the compact, "glandular" (Granel, 1922) pseudobranch (as in *Fundulus*—Copeland and Dalton, 1958; Ritch and Philpott, 1969) and the "free" pseudobranch resembling a gill hemibranch (as in the flounder—Harb and Copeland, 1969) are characterized by a cell type, the pseudobranchial cell, with an unique and elaborate ultrastructure.

TABLE V

Fish with well-developed choroid retia but weakly-developed swimbladder retia or oxygen secretion into the swimbladder.

		Reference to Swimbaldder
Herring	*Clupea harengus*	Fahlén, 1967a
Smelt	*Osmerus mordax*	Fahlén, 1968
Capelin	*Mallotus villosus*	Fahlén, 1968
Whitefish	*Coregonus clupeaformis*	Fahlén, 1967b Sundness, 1963
Brown trout	*Salmo trutta**	Wittenberg, 1958
Grayling	*Thymallus cthymallus*	Fahlén, 1968
Argentine	*Argentina silus*	Fänge, 1958
Carp	*Cyprinus carpio*	Fänge and Mattisson, 1956 Wittenberg, 1958
Goldfish	*Carassius auratus*	Wittenberg, 1958
Swordfish	*Xiphias gladius*	This Work

* The measured oxygen pressure in the eye of trout is large (Fairbanks, Hoffert and Fromm, 1969).

Pseudobranchs homologous (Goodrich, 1930; Müller, 1839) with that of teleosts are found in elasmobranchs and in the Actinopterygii, but are lacking in the Sarcopterygii (Goodrich, 1930). The pseudobranchs of *Amia* and of teleosts are distinguished from the foregoing by the presence of large granular acidophil cells which when characterized in the electron microscope may be called "pseudobranchial cells." The difference is sufficiently striking that Goodrich (1930, page 521) on the basis of observations in the light microscope, was led to conclude that the pseudobranch in teleosts and *Amia* seems "to have acquired a new function . . . in addition to its original function." Electron microscopic examination of the large pseudobranch of the gar, Lepisosteus, which lacks the choroid rete, reveals that pseudobranch type cells are absent (Harb, 1969). Regrettably, the pseudobranch of *Amia*, the other holostean, has not yet been studied in the electron microscope. We are here concerned with the interplay between the pseudobranch and the choroid rete and limit the discussion to *Amia* and the teleosts.

The position of the pseudobranch, astride the blood supply to the choroid rete mirabile, and the ultrastructural architecture of its cells, suggest that it may serve to modify the arterial blood entering the ophthalmic artery.

The pseudobranch may be well-developed in teleosts which lack choroid retia. This, however, is unusual, and of the fishes examined, we find it only in certain marine fishes, notably the Myctophidae, *Argyropelecus olfersi*, *Lepophidium cervinum*, and *Porichthys notatus*. In the myctophids, in particular, the pseudobranch is a conspicuous gill-like structure on the inside of the opercle. In our opinion, such an occasional situation indicates multiple functions for the pseudobranch. One such is known. The pseudobranch is proved to act as a chemoreceptor responsive to oxygen and carbon dioxide pressures of the incoming blood in tench (Laurent, 1967; Laurent and Dunel, 1964, 1966).

Every *marine* teleost (with exceptions noted) which lacks a pseudobranch also lacks the choroid rete. Instances are: many stomiatoids, some orphidioids and zoarcids and the deep-living arctic sculpin, *Cottunculus*. The apparent exceptions to this rule, *Opsanus, Lophius, Antimora* and *Halargyreus* can scarcely be considered to contradict it since the choroid rete in these forms is reduced almost to the vanishing point, and the oxygen pressure in the eye of *Opsanus* is not elevated (Wittenberg and Wittenberg, 1962, 1974).

All fish in which the choroid rete mirabile is well developed and in which the pseudobranch is absent, inhabit fresh water, Table VI. The Elopiformes, all of which have powerfully developed choroid retia, show a striking gradation in the size of the pseudobranch, Table VI. Those species which are primarily marine, *Elops, Albula, Dixonia* and *Pterothrissus*, have conspicuous, large pseudobranchs. The tarpon, *Megalops atlantica,* which lives at times in salt, brackish and fresh water, has a very small pseudobranch. Finally, the pseudobranch of the Australian species, *Megalops cyprinoides,* of which our specimens were captured in fresh water, and whose habitat may be confined to fresh water, is minute. Only a few stubby protuberances occupy the position where the pseudobranch would be found. We infer that a function of the pseudobranch which is required for the operation of the choroid rete mirabile may be dispensed with in fresh water.

We turn to the role of the pseudobranchs in oxygen secretion. The pseudobranch is not essential for oxygen secretion *per se,* neither in the swimbladder nor in the eye. Many fishes, for instance, the eels, *Anguilla, Conger, Muraena, Synaphobranchus,* the Notacanthiformes (McDowell, 1973), the midshipman, *Porichthys,* the toadfish *Opsanus,* some stomatiatoids, *e.g., Vinciguerria* and *Astronesthes,* and probably also morids, ophidiids and macrurids have swimbladder oxygen secretion powerfully developed although they lack pseudobranchs. (Bilateral extirpation of the pseudobranch was found to depress gas secretion into the swimbladder of *Fundulus* (Copeland, 1951) but not of *Perca* (Maetz, 1956), whose larger size may have made pseudobranchectomy a less traumatic procedure.) In a fresh water fish, the trout, *Salmo irideus*, the prior passage of blood through the pseudobranch is not necessarily required for oxygen secretion into the eye. A small area of the choriocapillaris is supplied with blood from an accessory counter-current exchange structure, the lentiform body, which receives blood from the general circulation by way of the retinal artery (Barnett, 1951).

The blood supply to the major counter-current exchange structure, the choroid rete, can be cut off by extirpation of the pseudobranch: the oxygen pressure in the ipsilateral eye nevertheless remains significantly elevated above arterial oxygen pressure, through action of the lentiform body (Fairbanks, Hoffert and Fromm, 1969).

TABLE VI

Relation of the pseudobranch to the choroid rete mirabile

		Pseudobranch	Habitat
	Order Elopiformes		
Ladyfish	*Elops saurus*	Large	Seawater
Machete	*Elops affinis*	Large	Seawater
Tarpon	*Megalops atlantica*	Very small	Brackish to fresh
	Megalops cyprinoides	Minute	Freshwater
Bonefish	*Albula vulpes*	Large	Seawater
	Dixonia nemoptera	Large	Seawater
	Pterothrissus gissu	Large	Seawater
	Order Osteoglossiformes		
Aruana	*Osteoglossum bicirrhosum*	Absent	Freshwater
Arapaima	*Arapaima gigas*	Absent	Freshwater
	Heterotus niloticus	Absent	Freshwater
	Scleropages guentheri	Absent	Freshwater
Butterflyfish	*Pantodon buchholzi*	Absent	Freshwater
Goldeye	*Hiodon alosoides*	Absent	Freshwater
Featherback	*Notopterus notopterus*	Absent	Freshwater
Featherback	*Noropterus chitala*	Absent	Freshwater
African knifefish	*Xenomystus nigri*	Absent	Freshwater
	Order Salmoniformes		
	Suborder Esocoidei		
Mudminnow	*Umbra limi*	Absent	Freshwater
	Order Channiformes		
Snakehead	*Ophicephalus micropeltes*	Absent	Freshwater
Snakehead	*Channa asiatica*	Absent	Freshwater
	Order Perciformes		
Climbing perch	*Anabas oligolepis*	Absent	Freshwater
Gourami	*Helostoma temmincki*	Absent	Freshwater

We seek to discover the special constraints on the functioning of the choroid rete which require the presence of the pseudobranch. We note that the choroid rete secretes into a fluid-filled space, and any materials there concentrated must remain, except as they are removed by ancillary vascular circulation. The gas space of the swimbladder, on the other hand, allows transient accumulations of gases to be diluted by expansion into its volume. In many species an elaborate accessory circulation carries away transient and steady state accumulations of carbon dioxide and other gases.

We focus attention on the gas carbon dioxide. The counter-current system of the choroid rete mirabile, acting as a counter-current multiplier and while secreting oxygen, in theory, must also accumulate carbon dioxide at its distal end. The theory of countercurrent multiplication in this system, proposed by W. Kuhn and his associates (Kuhn and Marti, 1966; Kuhn, Ramel, Kuhn and Marti, 1963), and recently somewhat amended (Alexander, 1966; Berg and Steen, 1968, Wittenberg and Wittenberg, 1974), is discussed by Wittenberg, Schwend and Wittenberg (1964), as it applies to carbon dioxide. During oxygen secretion, the carbon dioxide pressure in equilibrium with the capillaries at the distal end of a swimbladder rete reaches 275 mm Hg or 0.37 atmosphere (Wittenberg *et al.*, 1964). If a similar situation were to occur in the eye, the concentration of carbon dioxide near the retina would be 13.5 millimolar [At 20° C and assuming the solubility of CO_2 in eye tissues is similar to that of blood plasma (Van Slyke, Sendroy, Hastings and Neill (1928).] At physiological pH, the carbon dioxide would be 90 per cent or more ionized, and the protons so generated might well overwhelm the local buffering capacity and be damaging to the tissue. We advance the suggestion that the pseudobranch acts to modify the opthalmic arterial blood so as to prevent the accumulation of excessive carbon dioxide at the distal end of the choroid rete.

This suggestion immediately encounters a difficulty. In some species the pseudobranch is buried, and the degree to which it has access to ambient water, different in different species, is not yet established. Nonetheless there is strong evidence that buried pseudobranchs do act to modify the ionic composition of the ophthalmic arterial blood. The activity of sodium-potassium-activated adenosine triphosphatase (an enzyme implicated in ionic transport) is enhanced tenfold in the buried pseudobranch of *Fundulus* maintained in sea water relative to the fresh water controls (Epstein, Katz and Pickford, 1967). Conceivably material exchange in the buried pseudobranch occurs, not with seawater, but with the systemic blood of surrounding tissues. We put this difficulty aside for the moment, and return to the question of what material may be exchanged.

Total removal of carbon dioxide and bicarbonate anion from the ophthalmic arterial blood will not do, although decreasing their concentration might be helpful. An essential point in the operation of the countercurrent multiplier is the conservation of protons generated within the system. Carbon dioxide is required to serve as a mobile carrier of protons across the capillary wall from the outgoing to the incoming blood stream.

Secondly, the chemical composition of the blood of fresh and salt-water fishes (Holmes and Donaldson, 1969) gives no clue as to why fresh water fish alone may dispense with the pseudobranch. The total carbon dioxide contents of the blood of fresh and salt-water teleosts fall within the same range (4–10 millimoles per liter).

An attractive possibility is that the pseudobranch might effect a forced exchange of blood bicarbonate ion for external chloride ion. Such a process is described by Maetz and Romeu (1964) (Romeu and Maetz, 1964) in the gill of the goldfish; it depends on carbonic anhydrase. The pseudobranch of a marine fish carrying out a bicarbonate/chloride exchange could reduce total blood carbon dioxide to a level which when multiplied in the counter-current system would not be damaging.

In fresh water, such exchange would be limited by the available chloride, and the pseudobranch could be dispensed with, as it has been in some groups.

We discuss next the role of carbonic anhydrase in oxygen secretion. Carbonic anhydrase occurs in massive concentration in all structures involved in oxygen secretion. These include the pseudobranch (Hoffert, 1966; Leiner, 1940; Leiner and Leiner, 1940; Maetz, 1956; Sobotka and Kann, 1941), the choroid rete mirabile (Hoffert, 1966; Leiner, 1939, 1940; Leiner and Leiner, 1940; Maetz, 1956), the pigment cell layer of the teleost retina (Leiner, 1939; Maetz, 1956), the swimbladder rete mirabile (Fänge, 1950, 1966; Maren, 1962), and the gas gland of the swimbladder (Fänge, 1953). Significantly, the pseudobranch of elasmobranchs, which have no choroid rete, lacks this enzyme (Leiner, 1939).

The specific inhibitor of carbonic anhydrase, diamox, when administered to trout, *Salmo gairdneri,* abolishes active secretion of oxygen into the eye (Fairbanks, Hoffert and Fromm, 1969). Gas secretion into the swimbladder is also depressed by inhibitors of carbonic anhydrase (Fänge, 1950, 1953; Maetz, 1956). The primary actions of the carbonic anhydrase inhibitors cannot be other than multiple, effecting each of the array of structures involved in gas secretion and in addition effecting also the kinetics of gas exchange in the red blood cells (Berg and Steen, 1968) both at the gill and at the two retia. Lactate production by the swimbladder gas gland is inhibited by inhibition of carbonic anhydrase (Kutchai, 1971). Furthermore, it should be borne in mind that carbonic anhydrase accelerates not only the reaction for which it is named but is involved also in transcellular transport of ions (Keynes, 1969; Maren, 1967a, 1967b; Carter, 1972). The effect of the loss of carbonic anhydrase activity on the ionic environment of the retina may be distinguished from the many other effects, following the penetrating analysis developed by Maren (1967b).

Inhibition of carbonic anhydrase of the red blood cells markedly slows carbon dioxide exchange at the gill and thereby more than doubles the partial pressure of carbon dioxide in the blood (Hoffert and Fromm, 1966; Maren, 1967b). Since the gas diffuses freely through tissue, the pCO_2 is everywhere increased. The events in the absence of counter-current exchange or multiplication may be inferred from the events at the ciliary body, a structure whose epithelium shares a common embryological origin with the pigment cell epithelium of the retina. Diamox inhibits formation of bicarbonate ion from blood-borne carbon dioxide at the ciliary body (Maren, 1967b); the bicarbonate concentration of the aqueous is thereby diminished (Hoffert and Fromm, 1966; Maren, 1962), and as already noted, the partial pressure of carbon dioxide is elevated. The result is a more acid pH. In the trout, the change is from a normal value of pH 7.65 to pH 7.22 (Hoffert and Fromm, 1966).

The counter-current system of the choroid rete mirabile would be expected to multiply a locally generated increment in carbon dioxide pressure many fold. This increment, the "single concentrating effect" (Kuhn and Marti, 1966; Kuhn, *et al.*, 1963), at least in the swimbladder rete, is the generation of carbon dioxide from blood borne bicarbonate anion (Wittenberg *et al.*, 1964) through the action of lactic acid added to the blood locally. The amount of blood bicarbonate ion available may limit the magnitude of the increment (Wittenberg *et al.*, 1964). If diamox, by interfering with pseudobranchial function, causes the local blood bicarbonate ion concentration to rise, the carbon dioxide pressure generated by the "single concentrating effect" will also rise. The counter-current multiplication effect in the choroid rete mirabile would be expected to multiply this larger pressure increment several fold with a consequent large fall in pH, at the distal end of the system. This expectation is borne out in a crucial experiment reported by Maetz (1956), who measured the pH of the vitreous humor adjacent to the retina of perch. Normally the pH of the vitreous is pH 7.6, slightly alkaline to the blood plasma (pH 7.45). Following administration of diamox, the pH of the vitreous fell precipitously, more than 0.5 unit, to pH 6.9–7.1 within an hour. In the ensuing twelve hours, it fell nearly as much again, to reach pH 6.6. This acidity was sufficient to destroy the retina, and the animals became blind. The pH is that expected of a weakly buffered, strong solution of carbonic acid. The pressure of carbon dioxide must be very elevated.

In this experiment, diamox accentuated an effect inherent in the operation of a counter-current system, multiplication of the concentration of the diffusible carbon dioxide which, when hydrated, becomes a strong acid. Maetz's result strengthens our contention that the pseudobranch acts in consort with the choroid rete mirabile to create a high oxygen concentration at the retina without simultaneously accumulating an excessive concentration of carbon dioxide.

Thus, our findings combined with those of others lead to a working hypothesis for a function of the pseudobranch. A virtue of the hypothesis is that it may be put to the test of experiment. That experiment is to measure and compare ionic exchanges in the pseudobranchs of marine and fresh-water fishes.

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SUMMARY

The choroid rete mirabile is a vascular counter-current organ located behind the retina of the eye and responsible in part for the maintenance of a high partial pressure of oxygen there. It is absent in cyclostomes, elasmobranchs, and all living

non-teleost bony fishes with the exception of the holostean, *Amia calva.* The choroid rete is found widely distributed among teleosts and is nearly always present in the Acanthopterygii, which comprise the great majority of living forms. The ability to do without a choroid rete typifies families or orders, but is a character of limited phyletic usefulness. There seems little correlation between habitat and presence or absence of the choroid rete. The choroid rete and the rete mirabile of the swimbladder occur independently. This does not seem to be true for the choroid rete and the pseudobranch, since almost all fishes which have a choroid rete also have a pseudobranch. Arterial blood comes to the choroid rete mirabile by way of the pseudobranch, and those instances in which the latter is lost offer an occasion for deductions about its function. We argue that the pseudobranch acts to modify the incoming arterial blood in such a way that the choroid rete may concentrate oxygen without simultaneously concentrating carbon dioxide, which when hydrated becomes a strong acid.

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THE

BIOLOGICAL BULLETIN

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OXYGEN CONSUMPTION OF AN ASTEROID AND AN ECHINOID FROM THE ANTARCTIC

Reference: *Biol. Bull.*, **146**: 157–164. (April, 1974)

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A number of measurements of oxygen consumption by temperate and warm-water echinoderms are now available (Steen, 1965; Farmanfarmaian, 1966; Giese, Farmanfarmaian, Hilden and Doezema, 1966; Giese, 1967; Johansen and Vadas, 1967; Lewis, 1967; McPherson, 1968; Johansen and Peterson, 1971; Webster, in preparation; Webster and Giese, in preparation), but no studies have been recorded for polar forms. It is of great interest, therefore, to compare oxygen consumption of echinoderms living at near the freezing point of water to those living at temperatures of 10 to 25° C in temperate and tropical waters. An opportunity presented itself to make such studies on echinoderms from the antarctic waters off McMurdo Station, where the sea temperature is almost constantly −1.8° C.

The investigation described here deals with aspects of the respiratory physiology of the echinoid *Sterechinus neumayeri* and the asteroid *Odontaster validus,* with special emphasis on the contribution of the body wall to the oxygen consumption of the intact organism. The effects of small temperature increases on the oxygen consumption of these species were also examined.

MATERIALS AND METHODS

Odontaster and *Sterechinus* are conspicuous components of the shallow marine benthic community in the antarctic, especially near McMurdo Station (Dayton, Robillard and Paine, 1970). Both were collected by hand while diving on SCUBA near Cape Armitage, McMurdo Sound, Antarctica (79° S, 166° E) during October and November 1972. All animals were obtained in less than 20 meters of water. Following collection, the specimens were transported in insulated containers to the Eklund Biology Laboratory at McMurdo Station, where they were placed in refrigerated seawater aquaria held at −1.8° C. While the animals were not fed, diatoms and other detrital material were probably available in the aquaria.

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TABLE I

Body components*		*Sterechinus*	*Odontaster*
Body wall		28.9 ± 2.8	42.2 ± 5.4
Gonad	M	20.0 ± 10.1	4.6 ± 3.2†
	F	31.0 ± 14.4	—
Lantern		4.2 ± 0.9	—
Caeca		—	20.3 ± 2.5
Gut		2.9 ± 1.0	2.7 ± 0.7
Fluid	M	44.1 ± 10.9	29.4 ± 9.0†
	F	32.5 ± 13.4	—
Wet weight	g	71.6 ± 10.1	18.5 ± 5.9

* Averages for 15 specimens plus or minus one standard deviation; M = male; F = female. Values are given in percent of wet body weight.

† Sex not differentiated.

Oxygen consumption rates were determined for individual animals in closed respirometers with Clark-type oxygen electrodes. The respirometers consisted of glass containers surrounded by plexiglas cooling-jackets. Coolant (50% ethanol) was circulated through the cooling-jackets with a Forma-Temp Jr. regrigerated water bath. The experimental temperature was maintained within ±0.1° C. The respirometers were stirred with slowly revolving magnetic stirring bars, separated from the animals by plexiglas grids. All experiments were carried out in fresh seawater passed through #1 Whatman filter paper. Oxygen consumption was continuously monitored after the methods of Childress (1968). Each experiment lasted 4 to 8 hours, during which the partial pressure of oxygen fell from the maximal value to the minimal at which respiration could be measured.

The rate of oxygen consumption was determined for each species at three temperatures. For acclimation to temperatures above ambient (−1.8° C), the temperature of the aquarium in which the animals were held was raised at approximately 1° C every 24 hours. The animals were held at the new temperature no less than three days before experiments were begun.

As a control on microbial respiration, the animal was removed from the chamber at the end of each experiment, air-saturated seawater was added to replace its volume, and the rate of oxygen consumption by the wash water in the chamber was again measured for at least two hours. These control rates were negligible except at 3° C. In all cases, control rates of wash water were independent of oxygen tension and constant with respect to time. Where a control rate was measured, it was subtracted from the respiratory rate measured with the animal in the chamber to obtain the net respiration of the animal.

After each experiment, the animal was removed from the respirometer and returned to the seawater aquarium for no less than two hours. Following this, the animal was weighed and opened, and the gonads, gut, perivisceral fluid, caeca (stars), and lantern (urchins) were removed and weighed. The body wall was then placed in the respirometer, and oxygen consumption was determined as for the whole animal. Oxygen consumption by the wash water was determined for these experiments also.

The wet-weight measurements of whole animals and their component parts are approximations. Both species studied tend to lose fluid for a long period after

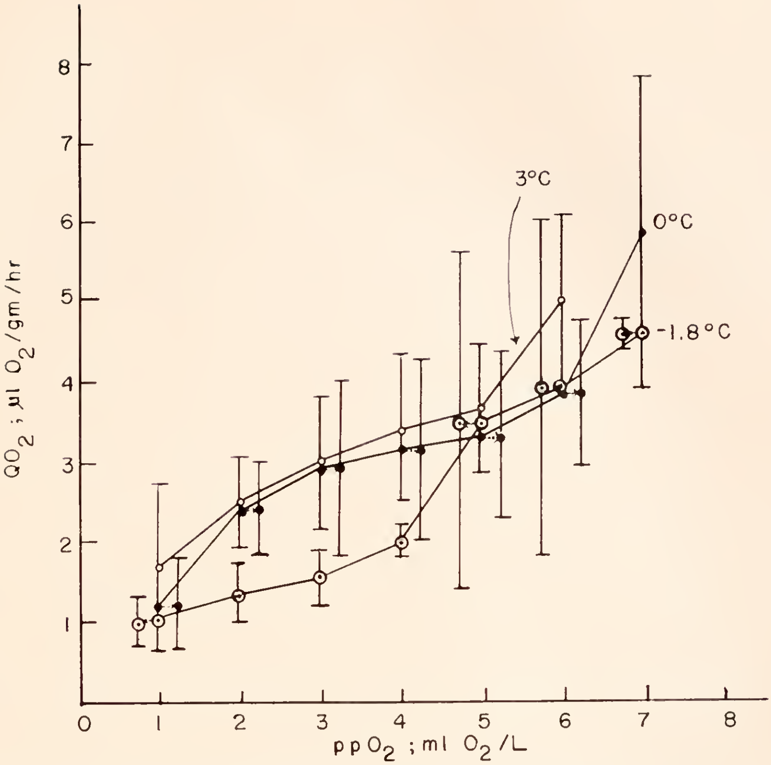

FIGURE 1. Oxygen consumption (QO_2, ordinate) in $\mu l O_2/g/hr$ for the echinoid *Sterechinus neumayeri* at −1.8° C, 0° C, and 3° C at different partial pressures of oxygen (ppO_2, abscissa) in $ml O_2/l$. Experiments lasted 4 to 8 hr in Figures 1 to 4. The bars about the means are single standard deviation units. The bars in Figures 1–4 for −1.8° C are offset to the left to avoid confusion.

removal from the water (Pearse, 1965). To standardize the weights used, the organisms were removed from the seawater and allowed to drain for five minutes on paper toweling before weighing, in accordance with the arbitrary choice commonly employed for echinoderms (Giese, 1966).

RESULTS

The measurements of body components of *Sterechinus* and *Odontaster* are summarized in Table I. The body wall constitutes 29% of the total mass of *Sterechinus* and 42% of *Odontaster*. These values must be considered only an order of magnitude, however, since, as noted above, these species lose water constantly and wet weights are approximations. The high gonad index, about 20% in male and 31% in female *Sterechinus* indicates that the sea urchins had not yet spawned-out for the year. Spawning usually occurs in this species during October and November (Pearse and Giese, 1966). Several specimens did, in fact, spawn during experiments (these experiments were discarded). The low gonad index of *Odontaster* corresponds to the index previously found at this time of year (Pearse, 1965). The caecum of *Odontaster* makes up for the bulk lost with spawn-out in the gonad.

Figures 1–4 show the rates of oxygen consumption of whole and eviscerated *Sterechinus* and *Odontaster* at three temperatures under conditions of falling ppO_2. Since the $\underset{\sim}{Q}O_2$ was measured in a "closed" system, the falling ppO_2 is due to the respiration of the animal. The proportional decrease in $\underset{\sim}{Q}O_2$ with decreasing ppO_2 is as expected, since echinoderms are generally considered to be "conformers" (Farmanfarmaian, 1966).

The mean per cent of saturation of dissolved oxygen in the water near McMurdo Station is 69%, with a range of only 6% (Tressler, 1964). Since the animals studied occur at this oxygen level, the mean $\underset{\sim}{Q}O_2$ for each was calculated from the data in Figures 1–4 at the corresponding ppO_2. For whole *Sterechinus*, the mean $\underset{\sim}{Q}O_2$ at −1.8° C is 3.5 ± 2.1 $\mu lO_2/g/hr$; for the body wall, 5.6 ± 0.9 $\mu lO_2/g/hr$. The mean oxygen consumption for the asteroid at −1.8° C is 4.9 ± 1.5 $\mu lO_2/g/hr$; for the body wall, 9.6 ± 2.0 $\mu lO_2/g/hr$.

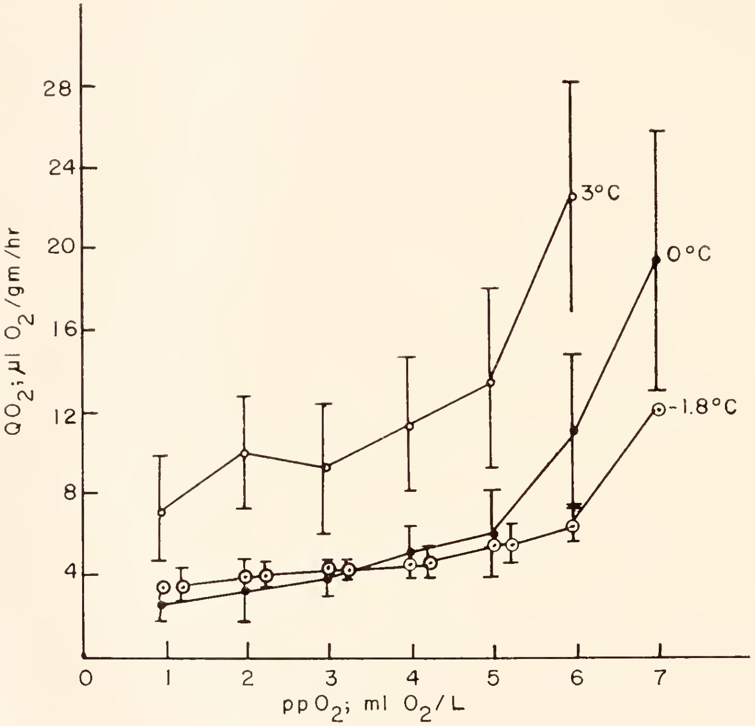

FIGURE 2. Oxygen consumption ($\underset{\sim}{Q}O_2$, ordinate) in $\mu lO_2/g/hr$ for the body wall of the echinoid *Sterechinus neumayeri* at −1.8° C, 0° C, and 3° C at different partial pressures of oxygen (ppO_2, abscissa) in mlO_2/l. The bars about the means are single standard deviation units.

There was no significant increase in $\underset{\sim}{Q}O_2$ with rise in temperature within the range tested (−1.8° C to 3° C) for either whole *Sterechinus* or *Odontaster*. For the body wall of the urchin, the QO_2 at 3° C was higher than at either 0° C or −1.8° C, the rates at the latter temperatures not differing significantly (Fig. 2). It was not possible to extend this study to a wider range of temperatures, because below −1.8° C the medium froze and at 5° C the animals became flaccid and eventually died.

It would be interesting to determine the effect of the reproductive state (*e.g.*, gonad index) upon oxygen consumption in these polar species as has been done for temperate species (Webster and Giese, in preparation). Animals of greatly differing gonad index were not available during the short period of time spent in the Antarctic. However, no significant correlation could be demonstrated between the rate of oxygen consumption and the gonad index by the coefficient of correlation test. No significant correlation between the rate of oxygen consumption and the gonad index was observed in the purple sea urchin *Strongylocentrotus purpuratus*, for which data are available spanning the entire reproductive cycle (Webster and Giese, in preparation).

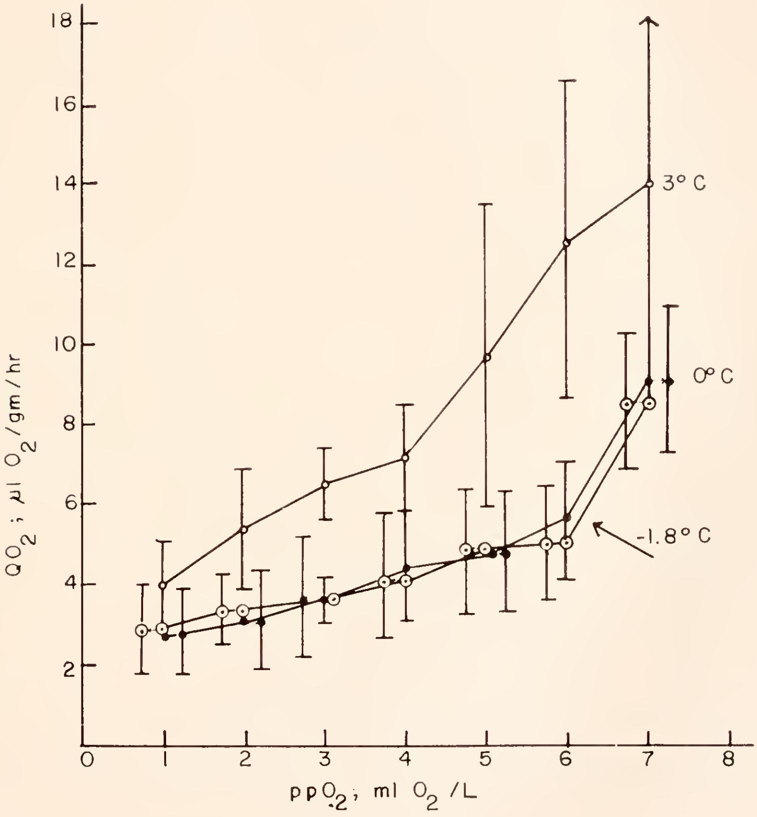

FIGURE 3. Oxygen consumption (QO_2, ordinate) in μlO_2/g/hr for the asteroid *Odontaster validus at* −1.8° C, and 3° C at different partial pressures of oxygen (ppO_2, abscissa) in mlO_2/l. The bars about the means are single standard deviation units.

DISCUSSION

The mean rate of oxygen consumption, QO_2, of the antarctic sea urchin *Sterechinus* determined in the present study compares with the QO_2 of a number of sea urchins of temperate waters, for example *Strongylocentrotus franciscanus* of the west coast of North America at about 13° C, *Allocentrotus fragillis* from deeper waters off the same coast at 8° C, and two sea urchins, *Evechinus chloroticus* and *Goniocidaris umbraculum,* from New Zealand at 13° C. The QO_2 for *Sterechinus* is considerably less than that for various tropical sea urchins: *Lytechinus anamesus* from Punta Banda, Mexico at 16° C, *Echinometra mathaei* from Savai's, Western Samoa at 23° C, *E. lucunter* and *Tripneustes esculentus* from St. John, U. S. Virgin Islands at 28° C (Webster, in preparation).

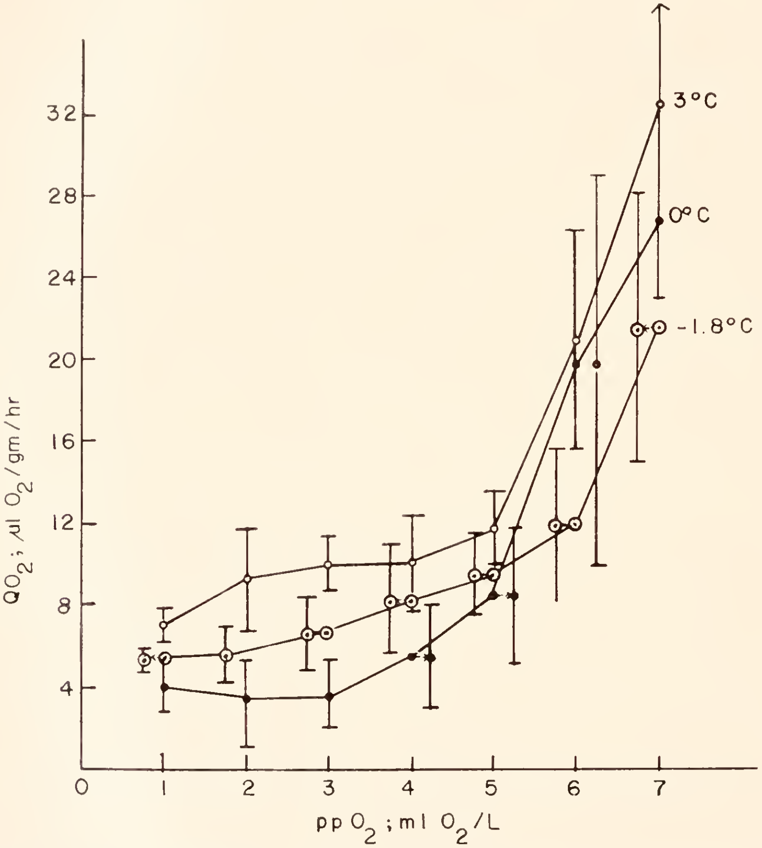

FIGURE 4. Oxygen consumption (QO_2, ordinate) in $\mu l O_2$/g/hr for the body wall of the asteroid *Odontaster validus* at −1.8° C, 0° C, and 3° C at different partial pressures of oxygen (ppO_2, abscissa) in $ml O_2$/l. The bars about the means are single standard deviation units.

That the body wall of *Sterechinus* is probably the main consumer of oxygen during respiration in the echinoid is not surprising in view of its prominence in the body and its biological nature (Table I). In all echinoderms, the body wall is not strictly a skeleton, but contains many cells, stores of nutrient, and some muscle and nerve. That the body wall should have a $\underset{\sim}{Q}O_2$ higher than the whole body is explainable in terms of the availability of oxygen to the internal tissues of the whole animal. In the intact purple sea urchin, the oxygen in seawater impinging on the outer surface is quickly used, so that the perivisceral fluid within has only one-half to one-fourth (or less) the oxygen at the surface (Webster and Giese, in preparation). The inner surface of the body wall of the echinoid is therefore bathed in fluid with a considerably attenuated oxygen supply. When the body wall is dissected out and the internal side is exposed to the same oxygen levels as the external side, the whole body wall receives much more oxygen than previously, and its QO_2 rises accordingly.

Much of what has been said of *Sterechinus* applies equally well to *Odontaster.* The whole body QO_2 of *Odontaster* is of the same order of magnitude as the QO_2 of *Zoroaster evermanni* and *Dermasterias imbricata* at 13° C, both from Monterey Bay, and *Pentagonaster pulcrellus* at 13° C from New Zealand. The $\underset{\sim}{Q}O_2$ of *Odontaster* is from $\frac{1}{2}$ to $\frac{1}{5}$ that of a series of six asteroids from Monterey Bay measured at 13° C (Webster, in preparation). We found no data on tropical asteroids with which to make comparisons. In *Odontaster* the body wall has a higher $\underset{\sim}{Q}O_2$ than the whole body, probably for the same reason as in *Sterechinus.*

The conclusion we have drawn from the data presented in Figures 1 and 3 is that temperatures up to 4.8° C above ambient have no significant effect on the rate of oxygen consumption of acclimated *Sterechinus* and *Odontaster.* It is possible, of course, that such an effect has been masked by the variability of individual experiments. The relatively small difference between the mean $\underset{\sim}{Q}O_2$ at each temperatures suggests, however, that, even if this were the case, the effect of temperature on the QO_2 in these species is slight. Therefore, over the range of temperatures $-1.8°$ C to 3° C, the temperature coefficient appears to be close to unity. Over this temperature range the metabolism of these antarctic echinoderms appears to be temperature-insensitive, suggesting metabolic rate compensation. Temperature coefficients for thermal acclimation over a wide range of temperature in the purple sea urchin suggest enzymatic adaptation (Farmanfarmaian and Giese, 1963). A study of the enzymatic basis of thermal acclimation on representative echinoderms from tropical, temperate and polar environments, similar to that made on other animals as recently reviewed (Hochachka and Somero, 1973), would therefore be of great interest.

This research was conducted under Antarctic NSF Grant #GA4458 to A. C. Giese, who planned the present study as part of a larger program on "The Physiology of the Body Wall of Echinoderms." Special thanks are given to David Checkly and A. L. DeVries who assisted in the diving program, to U. S. Navy Task Force 43 for logistical support, to Albert Towle of the State University of California, San Francisco, who in March 1970 did preliminary experiments on respiration of *Odontaster validus* which were useful in setting up the program for the present report, and to John Pearse of the University of California, Santa Cruz, for advice concerning collection and maintenance of both species studied.

SUMMARY

The mean oxygen consumption, QO_2, in $\mu lO_2/g/hr$ at $-1.8°$ C (ambient temperature), of the antarctic echinoid *Sterechinus neumayeri* and the antarctic asteroid

Odontaster validus, determined with an oxygen electrode, were recorded as follows: *S. neumayeri* (whole body) 3.5 ± 2.1, (body wall) 5.6 ± 0.9; and *O. validus* (whole body) 4.9 ± 1.5, (body wall) 9.6 ± 2.0. The oxygen consumption fell with the decline in partial pressure of oxygen as the oxygen was used by the organisms. Small increases in temperature, from −1.8° C to 3° C, had no significant effect on the $\underset{\sim}{Q}O_2$ of whole animals, and at 3° C only a small effect on the $\underset{\sim}{Q}O_2$ of the body wall. Comparisons indicate that some temperate species have $\underset{\sim}{Q}O_2$ values in the same range as the antarctic species. Tropical species cited, however, consume oxygen at a higher rate than the antarctic species.

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THE SEASONAL CYCLE OF COPPER CONCENTRATION IN *BUSYCON CANALICULATUM* L.

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Copper is present in seawater in only trace amounts, on the order of 3 μg/l (Goldberg, 1965). Nevertheless, the copper concentration in marine gastropods has been shown to be generally high, up to 100 μg/g (Marks, 1938; Vinogradov, 1953; Segar, Collins and Riley, 1971). This implies the presence of a mechanism for storing copper taken up either from the diet or directly from seawater. Riley and Segar (1970) have stated that for marine animals in general, "Little is known about the mechanisms by which trace elements are concentrated or about the manner in which they are held in tissues" (page 721).

Marine gastropods are known to use copper (in the synthesis of the blood pigment, hemocyanin), yet for no species has there been a description of copper distribution in the body complete enough to allow inferences to be drawn about either the extent of individual variation or the physiological and ecological factors which control it. In the present study, specimens of the locally abundant channeled whelk, *Busycon canaliculatum* L., have been examined for copper concentration in various organs and tissues at different seasons of the year.

MATERIALS AND METHODS

Approximately 100 specimens of *Busycon canaliculatum* L. were obtained from the region between Wickford and Fox Island in Narragansett Bay, Rhode Island, by collection in pots during the summer and fall and by dredging during the winter and spring and were held without feeding in running bay water until used. The weights of the animals used ranged in general from 100 to 300 g. Average tissue weight (total fresh weight minus shell weight) was 120 g.

Whelks were removed from the shell, and whole organs and tissue samples were dissected free and weighed. Blood samples were obtained by placing the body in a funnel, cutting into the foot muscle with a scalpel, and allowing blood from the pedal sinus to drain into a graduated centrifuge tube. Some samples of blood were obtained, prior to dissection, from a hole drilled in the operculum.

Blood samples were centrifuged and either analyzed immediately or frozen and stored for later analysis of protein and copper concentration. Protein was determined by the Biuret method (Layne, 1957) after 10- or 20-fold dilution with 0.1 M KCl. Bovine serum albumin was used as a protein standard.

Samples of blood and tissue were prepared for copper analysis by digesting with a solution made by mixing 100 ml of concentrated perchloric acid and 400 ml of concentrated nitric acid. The samples were placed in Pyrex® or Vycor® beakers or Kjeldahl flasks, with 5 ml or more of the acid solution, and heated gently until

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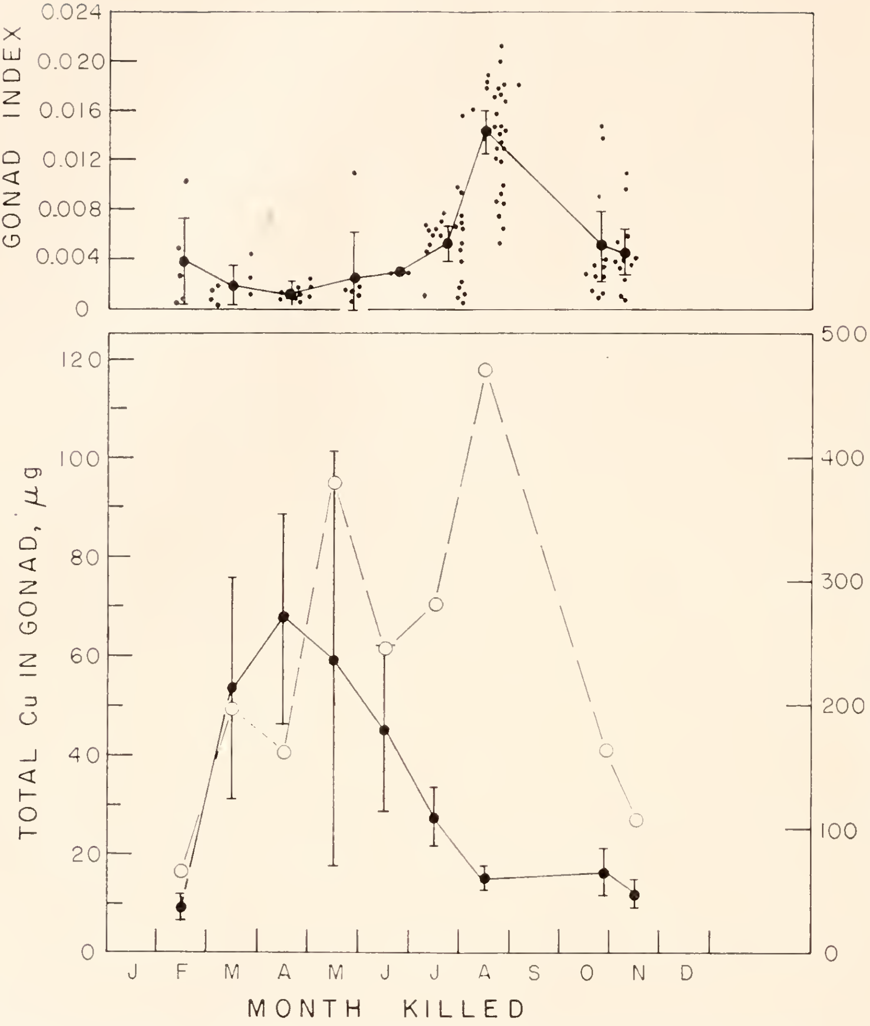

FIGURE 1. Annual changes in gonad index and gonad copper in *Buscycon*. Gonad index (fresh weight gonad/fresh weight whole soft tissues): points represent individual animals; closed circles, average gonad index for month; vertical bars represent two standard errors of the mean (S.E.M.) for month; open circles, monthly average total copper in gonad of 120-gram whelk (see text); closed circles, average copper concentration for month ± one S.E.M. Individual data on copper concentration which are summarized in Figures 1–4 are tabulated in Appendix IV of Betzer (1972).

all organic matter was oxidized and the nitric acid had boiled off. Ordinarily the digestive gland, gut, kidney, gonad, osphradium, and gill were each analyzed whole, while aliquots weighing 0.5–1.0 g were taken from other tissues (foot muscle, mantle, nidamental gland, oviduct or blood diluted with 0.1 M KCl). After digestion, the samples were analyzed either by atomic absorption spectroscopy or by the spectrophotometric cuproine method (B) of Diehl and Smith (1958). For atomic absorption analysis the samples were rinsed into volumetric flasks and made up to volume with deionized water, to give, where possible, a final copper concentration of 1–10 μg/ml. The solutions were run against double-deionized water on a Perkin-Elmer Model 303 atomic absorption unit, using a laminar flow burner with an air-acetylene flame. Samples were alternated with copper standards and run in triplicate. In analyses by the cuproine method, if a sample was thought to contain less than about 25 μg of copper, the whole sample was used for the analysis; samples containing greater amounts of copper were diluted with deionized water in volumetric flasks, and duplicate aliquots containing 5–15 μg of copper were analyzed. The cuproine method was modified by the addition of 5 g/l of hydroquinone to the cuproine reagent to retard fading of color (Riley and Sinhaseni, 1958). In the cuproine procedure, copper is extracted from the aqueous phase by combination with cuproine dissolved in isoamyl alcohol. It was found that better separation of water droplets from the organic layer in the final centrifugation step was achieved if the alcohol-cuproine solution was refrigerated before centrifugation. Standards (prepared from copper shot dissolved in reagent grade concentrated nitric acid and diluted with deionized water) and blank samples were prepared and analyzed in the same way as the tissue samples.

The average standard deviation for triplicate copper determinations by atomic absorption spectroscopy was 2.6% of the determined values. Cuproine determinations were ordinarily done in duplicate, and the average standard deviation calculated from duplicate aliquots from the same solution was 0.70% of the determined values; the average standard deviation calculated from duplicate samples of blood oxidized with acid in separate Kjeldahl flasks and then analyzed was 1.05% of the determined values. The average recovery of standards boiled with acid and then analyzed was 104% of the values for standards analyzed directly by the cuproine method. When the same samples were analyzed by both atomic absorption and cuproine methods, the average difference between results by the two techniques was 6.8% of the mean copper concentration.

The total body copper was determined in 6 whelks. Each was placed over a large watch glass during dissection to catch any blood or mucous running from the shell. After whole organ and tissue samples had been taken, the remaining whelk tissues were washed into a Waring Blendor with a known quantity of deionized water. The blender was run until the mixture appeared homogeneous, and then while it was still running a dropper was used to transfer aliquots to two Vycor® or Vitreosil® crucibles. All the crucibles were weighed to determine fresh weight of the tissue and then were dried overnight at 100° C and reweighed. They were then covered, placed in a muffle furnace, and ashed at 550° C for 8–20 h. After cooling, 1–2 ml of concentrated HCl was added to each crucible and allowed to stand about 1 h. The contents of the crucibles were then transferred with deionized water rinsing to volumetric flasks, for copper determination by the cuproine method.

RESULTS

Seasonal changes in copper concentrations in individual tissues

Busycon canaliculatum undergoes a seasonal cycle in the bay; during the warm summer months (beginning in late May or early June) whelks move about on the sediment, actively feeding, and are caught commercially in baited "pots." During the colder months, from late fall until late spring, the whelks lie buried in the sediment, no longer attracted by bait. The reproductive cycle shows a similar seasonal pattern, as can be seen from the annual changes in the gonad index (fresh weight gonad/fresh weight whole soft tissues) (Fig. 1). From this graph it appears that spawning may occur primarily in the late summer and fall, although occasional ripe individuals (having a gonad index greater than 0.002–0.003 and a

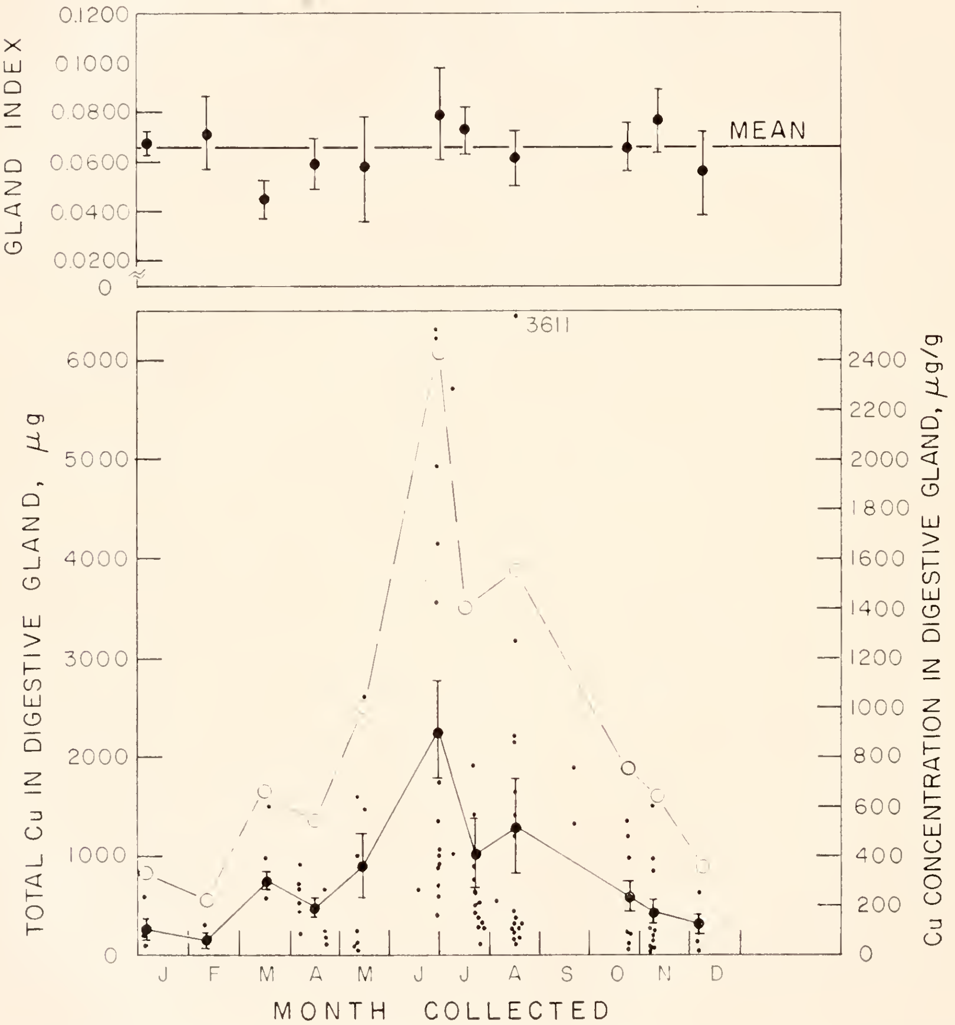

FIGURE 2. Annual changes in digestive gland index and digestive gland copper in *Busycon*. Digestive gland index (fresh weight digestive gland/fresh weight whole soft tissues): closed circles, average index for month ± two S.E.M.; open circles, monthly average total copper in digestive gland of 120-gram whelk (see text); closed circles, average copper concentration (μg/g) for month ± one S.E.M.; points represent concentrations in individual digestive glands.

well-developed penis or nidamental gland) are found in the spring. Relative weights (or "indices") of the other tissues did not show seasonal changes (*e.g.*, Fig. 2) except for a possible fall increase in the kidney (Fig. 4).

The copper concentrations in the tissues of *Busycon* were characterized by a high degree of individual variation, even in animals captured at the same time. This variability is illustrated in the plots of individual concentrations of copper in the digestive gland and blood (Figs. 2 and 3) and by the standard errors of the monthly means plotted for the gonad and kidney (Figs. 1 and 4). Despite the striking variability, clear seasonal patterns of copper concentration are evident for most tissues analyzed. All concentrations are reported in terms of μg Cu/g fresh weight. Total copper in each individual tissue of each whelk was normalized to that in an hypothetical "average whelk," using as a multiplication factor the ratio of 120 grams to the actual whole soft tissue weight. Monthly averages of these totals are plotted for the gonad, digestive gland, and kidney (Figs. 1, 2, and 4).

The digestive gland (= "hepatopancreas" or "liver") and gut showed the highest copper concentrations of all tissues analyzed, with that of the digestive

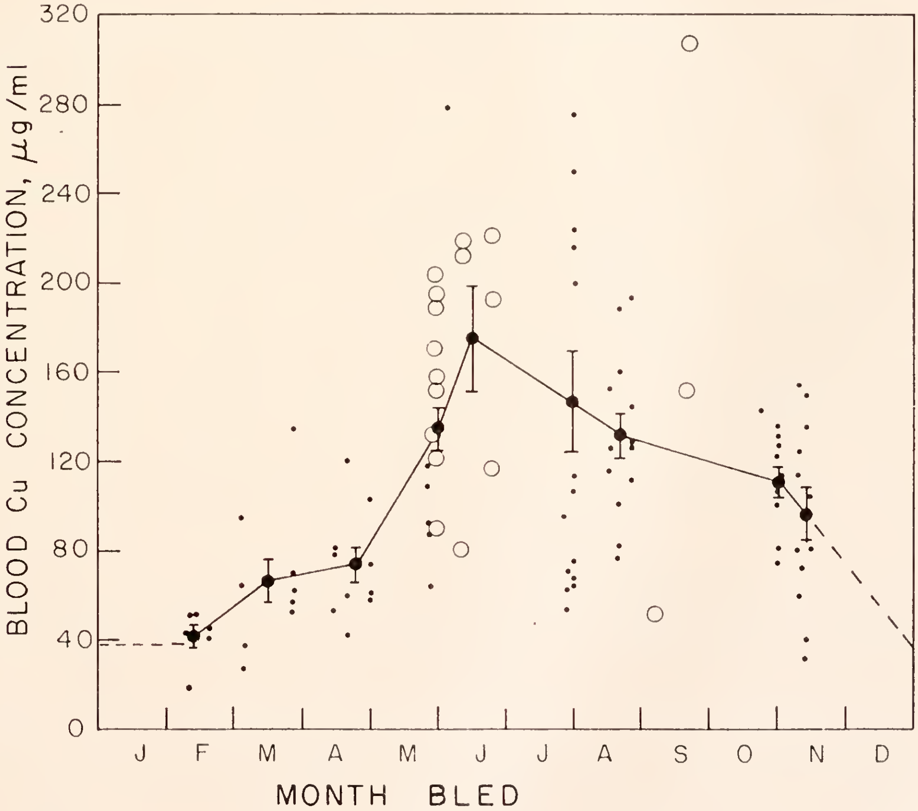

FIGURE 3. Annual changes in copper concentration of the blood of *Busycon*; individual whelks bled by slashing foot; open circles, individual whelks bled by withdrawing blood through operculum; closed circles, average copper concentration for month ± one S.E.M.

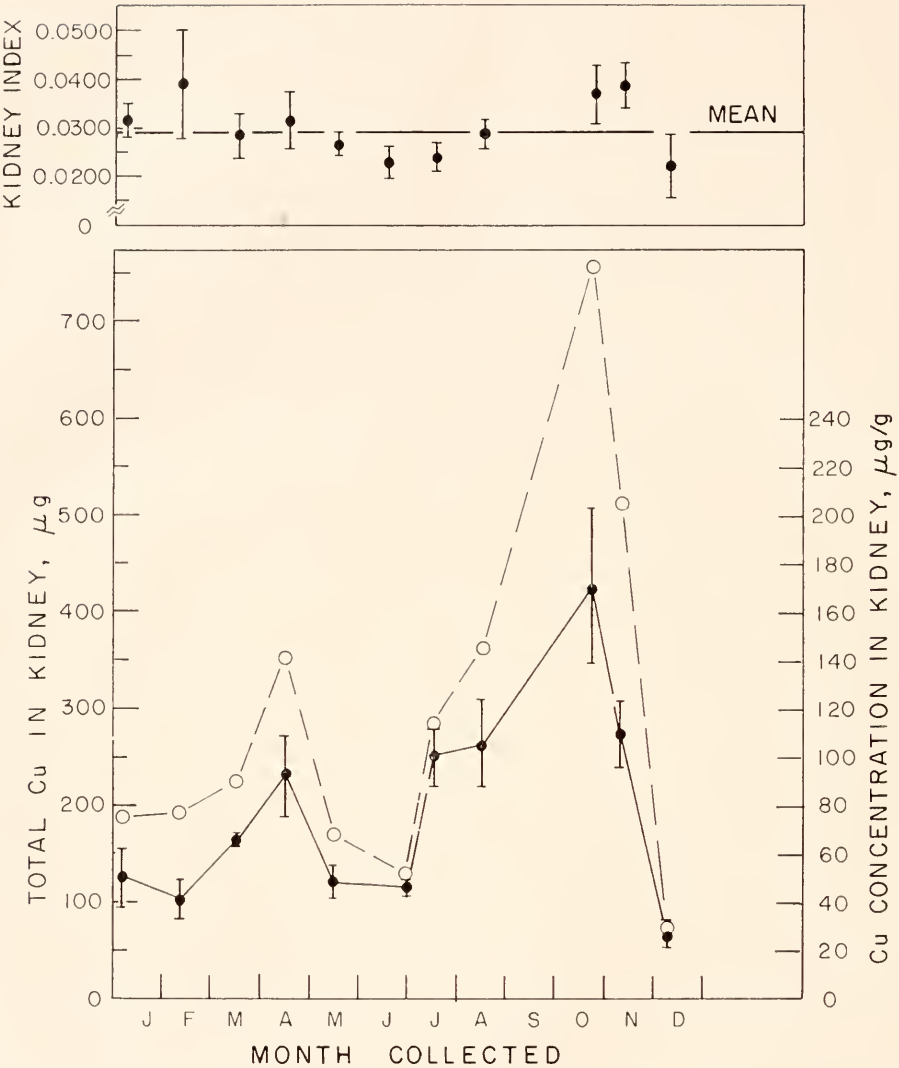

FIGURE 4. Annual changes in kidney index and kidney copper in *Busycon*. Kidney index (fresh weight kidney/fresh weight whole soft tissues); closed circles, average index for month ± two S.E.M.; open circles, monthly average total copper in kidney of 120-gram whelk (see text); closed circles average copper concentration ($\mu g/g$) for month ± one S.E.M.

gland being generally about twice that of the gut. The extreme values for the digestive gland were a low of 14 $\mu g/g$ in a whelk collected in December and a high of 3611 $\mu g/g$ in one collected in August. Values reported by Vinogradov (1953) for the digestive gland of other gastropods are low in comparison to the concentrations found during most of the year in *Busycon: Helix pomatia*—19 $\mu g/g$, *Purpura lapillus*—14 $\mu g/g$, and *Patella vulgata*—20 $\mu g/g$. Townsley's (1954) unpublished determinations of the copper concentration of small subsamples of the gut and digestive gland of *Busycon* fell within the range presented here, although he separated the gland into a "liver" and "pancreas" portion and found that the gut had a higher copper concentration than either of these portions.

In the large number of digestive glands analyzed in the present study, there was a seasonal trend, with low copper concentrations in the winter and early spring (monthly means of 65–120 μg/g), higher values in May and June (when the whelks emerge from winter hibernation), and a maximum (marked by considerable variability) in the summer (monthly means of 400–900 μg/g); copper concentrations were found to decrease again in the fall. The digestive gland index remained approximately the same throughout the year (Fig. 2); thus the 5- to 10-fold difference between midwinter and midsummer concentrations is reflected in changes in the total amount of copper in the digestive gland (Fig. 2). These changes were paralleled by changes in gut copper concentration, and the increased levels of copper presumably occurred when the whelks began to feed in the spring.

The seasonal pattern was quite different in the gonad, the organ next highest in copper concentration (Fig. 1). Here the copper concentration increased in early spring, reached a maximum in April, and steadily decreased from May to a low in midsummer, remaining low and fairly constant until the March increase. Because of concomitant increases in the gonad index, however, total copper in the organ actually increased 6-fold during the summer as spawning approached, even while the copper concentration decreased. By late fall, total copper had again decreased to winter levels.

In the blood of *Busycon*, copper is present as part of the metallo-protein, hemocyanin. For blood samples from 104 whelks, blood copper showed a linear relation to blood protein ($r = 0.97$): Cu (mg/ml) = 0.00235 protein (mg/ml)—0.0064. The copper averaged 0.235% (by weight) of the total protein in centrifuged blood; this is close to the concentration of 0.245% in purified *Busycon* hemocyanin (Hernler and Phillipi, 1933, cited in Redfield, 1934). This close agreement and small negative y-intercept suggest that only a small amount of the protein in the blood is not associated with copper (averaging 2.8 mg/ml); the presence of only this small quantity of non-hemocyanin protein is consistent with Ghiretti's (1966) statement that 90% of total blood protein in *Busycon* is hemocyanin. The consistent linear relationship between copper and protein and the good agreement with the literature value for the percentage of copper in *Busycon* hemocyanin provide additional evidence for the accuracy of copper determinations in the present study.

The relation between blood copper and protein did not change over the year; but their concentration showed great variability (Fig. 3), with extreme values of 19 μg Cu/ml (in February) and 316 μg Cu/ml (in July). This 17-fold variation in copper (and hemocyanin) concentration is much greater than the two-fold range in *Busycon* hemocyanin concentration reported by Redfield, Coolidge, and Hurd (1926) and Townsley (1954), perhaps because these investigators each sampled at a single season of the year. The large variation found even among whelks bled at the same time in our study was not correlated with sex or body weight.

The concentration of blood copper (and hemocyanin) rose and fell at roughly the same times of year as in the digestive gland and gut (although the copper concentration of the blood and digestive gland showed no direct relationship in individual whelks). In February, the average was 42 μg Cu/ml, rising sharply at the time of emergence from hibernation in May and June to a maximum of 175 μg/ml (Fig. 3), or about four times the winter level. There was a gradual decrease in concentration in the fall.

The seasonal pattern of copper concentration and total copper in the kidney (Fig. 4) was different from that of the other copper-rich tissues of the visceral mass. This organ was for most of the year comparable to or lower in copper concentration than the blood, but in the late summer and early fall, when the blood, digestive gland, and gut were decreasing in copper concentration, the kidney reached a maximum concentration (averaging 171 μg/g), exceeding the copper concentration of the blood. Total copper increased even more sharply, because of a larger kidney index. It then decreased to low values in the winter and spring. In a few whelks dissected in late summer and fall, the kidneys were noted to be dark blue instead of their usual brown color; on analysis these kidneys were found to have unusually high copper concentrations, and total copper (amounting to 1090 μg, 1895 μg, and 1137 μg in three kidneys) even exceeded that of the digestive gland. This pattern suggests that perhaps the kidney may have a role in excreting some of the copper accumulated over the summer. That the blood has not yet at this time decreased to its average winter copper concentration is consistent with a role in transporting tissue copper to the kidney for excretion.

Several other tissues were routinely analyzed—a sample of foot muscle, the whole osphradium, and the whole gill. The first two were generally very low year round and showed probably only random variation, the foot muscle averaging less than 20 μg/g for every month sampled, and the osphradium always about 40 μg/g or less. The gill, an organ richly supplied with blood, followed a pattern of copper concentration that paralleled that of the blood, ranging from an average of 15–30 μg/g in the winter to 70–80 μg/g in the summer. Probably the blood present in all three of these tissues accounted for the bulk of the copper.

Copper concentration of entire soft tissues

Six whelks captured over the course of a summer (May 15–Sept. 20) were analyzed for the copper content in total body tissues (Table I). The copper concentration ranged from 58–116 μg/g, with a mean of 76 μg/g; this is at the high end of the range of values collected by Vinogradov (1953) for other marine gastropods: *Haliotis crachcrodii*—0.8 μg/g, *Littorina*—4 μg/g, *Murex*—21 μg/g, and *Buccinum*—78.5 μg/g. The total body copper concentrations in three species that can be calculated from the data of Segar *et al.* (1971) are also lower than found here in *Busycon: Patella vulgata*—1.4 μg/g, *Buccinum undatum*—40 μg/g, and *Crepidula fornicata*—40.5 μg/g. The digestive gland contained the bulk of the tissue copper in these summer whelks, ranging from 47–82% of the total, with an average of 60%.

In the whelks analyzed there was no apparent relationship between the size of the animal and its copper concentration. For the copper determinations on both these animals in which entire soft tissues were analyzed and also those in which only individual organs were analyzed, some whelks taken in the summer were dissected within a few days of capture; others were kept without feeding up to 8 weeks before dissection. There was thus considerable variation in the period which had elapsed since the last possible feeding, but this had no apparent effect on the copper content of the whole whelk, digestive gland, or blood, or on the fraction of copper in the digestive gland. This suggests that, even if the diet is the main source of copper, frequent feeding is not necessary to maintain the high body copper concentrations found, and, in addition, that copper stored in the digestive gland is not rapidly depleted. Apparently, once copper has been accumulated in the late spring or early summer, it is not quickly turned over or excreted.

DISCUSSION

The high degree of variability in copper concentration found in *Busycon* tissues, particularly the digestive gland and blood, is less than was found for *Haliotis* blood (Pilson, 1965) but is similar to that noted by Rocca (1969) for the hepatopancreas of *Octopus vulgaris*. He found no significant correlation between the copper concentration of the digestive gland and the season when the animals were killed and suggested that the unusually high variability might be due to its role in copper absorption and storage in this animal. Weischer (1965) found seasonal variation as well as pronounced individual scatter in the blood and hepatopancreas copper concentrations of *Helix pomatia*, the terrestrial pulmonate snail; that of the hepatopancreas was highest in the summer, when the copper concentration of the blood was very low. In the fall, just before hibernation, the copper concentration in the blood climbed sharply and that in the hepatopancreas dropped. The blood copper concentration reached a maximum in January and was gradually reduced in the early summer. Weischer concluded that copper from the degradation of hemocyanin was stored in the hepatopancreas during the summer and used in autumn for renewed hemocyanin synthesis. The role, if any, for the very large reservoir of copper in the digestive gland of *Busycon* remains unknown.

TABLE I

Copper content of entire soft tissues of whelks and percentage Cu in digestive gland

Whelk no.	Sex	Tissue wt, g	Month captured	Days starved	Total Cu μg	μg Cu/g fresh wt	% of Cu in digestive gland
8	♂	31	May	55	1,801	58	47
9	♀	173	July	18	11,582	67	55
11	♀	220	Aug	9	15,484	70	70
16	♀	177	July	59	20,579	116	82
20	♂	54	Sept	33	3,618	67	52
21	♂	97	Sept	33	7,238	81	57
$\bar{x}$						76	60

The findings of both Weischer's and the present study tend to contradict Ghiretti's (1966) statement that in mollusks "during the life span of an animal the concentration of hemocyanin seems to remain fairly constant under normal physiological conditions" (page 234). In *Busycon*, unlike *Helix*, the copper concentrations in the digestive gland and blood do not vary reciprocally over the year;

instead, both tend to rise with the onset of feeding in early summer and to decrease in the fall. The differences in cycles of copper concentration in the tissues of the two species of snail may reflect differences in copper availability in their environments, as well as differences in their annual cycles of spawning and activity, and mode of hibernation. In *Helix*, blood copper is highest during hibernation; in *Busycon,* it is lowest during this period. *Busycon* has abundant copper available in the diet and lives bathed in a copper-containing solution: *Helix* has more limited access to the metal and so perhaps exercises greater control over storage and reuse of its supply. From neither of these studies can firm conclusions be drawn as to the primary function of hemocyanin in the snails; the evidence of seasonal variation could be used to support either a respiratory role or a role as a food (energy) reservoir, although the extreme variability argues against a primarily respiratory function (Pilson, 1965). Both studies underline the potential errors inherent in reports of observations of metal concentrations from small numbers of individual invertebrates without regard to season or physiological state of the animals.

We wish to thank Richard Sisson, of the Rhode Island Department of Natural Resources, and Stanley Spink for providing specimens of *Busycon.* Robert Duce kindly made available his Perkin-Elmer 303 atomic absorption spectrophotometer. Peter Betzer helped with copper analyses and with preparation of the manuscript. This work was done at the Graduate School of Oceanography of the University of Rhode Island while one of us (S. B. B.) was supported by a National Science Foundation Graduate Fellowship.

SUMMARY

Individual tissues of approximately 100 whelks were analyzed for copper content. Tissue copper concentrations showed a high degree of individual variation, but seasonal trends were evident. In the digestive gland, the organ with the highest copper concentration, the mean monthly concentrations were about 5 to 10 times higher in the summer (400–900 μg Cu/g fresh weight) than in the winter (65–120 μg Cu/g). Gut concentrations followed the same seasonal trend. The copper concentration in the gonad increased in the early spring and then decreased in the late spring and summer to low fall and winter values. The total copper in the gonad increased 6-fold during the spring and summer, however, because of large increases in size as late summer spawning approached. The total copper in the gonad dropped off after the spawning season. Monthly average blood copper and protein concentrations followed the same seasonal pattern as the digestive gland and gut, with copper ranging from a monthly average of 42 μg/ml in February to 175 μg/ml in June. Nearly all the blood protein present was hemocyanin, and there was no apparent seasonal change in the ratio of copper:protein. The copper concentration of the kidney was similar to that of the blood during most of the year, but in late summer and fall it increased to a maximum (171 μg/g in Oct.), while that of the blood and other tissues was decreasing. Whelks in Narragansett Bay usually begin to feed in late May or early June; the general early summer increases in the copper concentration of most tissues correlated with this commencement of feeding.

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Reference: *Biol. Bull.*, **146**: 176–185. (April, 1974)

GENETIC MOSAICISM IN THE WINGS OF *HABROBRACON JUGLANDIS*

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Work in *Drosophila* has shown that cell lineage patterns in wings, legs, and antennae are not random, but that clonally related cells appear in long narrow stripes in a longitudinal direction (Bryant and Schneiderman, 1969; Bryant, 1970; Postlethwait and Schneiderman, 1971). In *Drosophila* these patterns have been demonstrated by means of genetic mosaics which arose as a result of somatic crossing-over or of ring-X chromosome elimination. Mosaics have been found in the wasp, *Habrobracon,* over the past 40 years (P. W. Whiting, 1932; A. R. Whiting, 1939, 1961) but had not been studied with respect to lineage patterns. The discovery that the mutant *ebony* in *Habrobracon* is related to both a black body color and to the production of mosaics has enabled us to plan specific experiments involving selected gene markers and to obtain large numbers of mosaics for pattern analysis (Clark, Gould, and Graham, 1971). Lineage patterns of a longitudinal type have been found for antennae and legs of *Habrobracon* (Clark, Petters, and Bryant, 1973). In the present study such patterns are reported for wings.

The two wing mutants studied here affect the shape and size of the primary and secondary wings. Investigation of the effects on wing growth and development for mosaic wings when there is an interaction of two genotypes is significant.

Materials and Methods

In the parasitic wasp, *Habrobracon juglandis,* haploid males are produced from unfertilized eggs, and diploids, both male and female, from fertilized eggs. The haploid males are thus of gynogenetic (maternal) origin while the diploids are of zygogenetic (biparental) origin. In addition, various types of mosaic progeny are produced (Clark, Gould, and Potts, 1968). The mosaic type of present concern is one in which part of the body is zygogenetic (biparental) and the other part is androgenetic (paternal) in nuclear origin (Z + A type).

Two recessive wing mutants were used to study patterns of development, *notched wings* (*no*) and *small wings* (*sw*). Notched wings is a temperature-sensitive mutant. Kershner (1970) showed for wasps reared at four different temperatures (35°, 30°, 25°, 18° C) that the size of the wing was dependent upon the temperature at which development occurred. In contrast to the wild type which showed only slight increases in size and no change in wing shape, the *notched wings* showed progressively smaller wings with lowered temperatures (Fig. 1). *Notched wings* reared at 35° C is phenotypically indistinguishable from wild type reared at the same temperature. At lower temperatures there is a progressive loss of the distal portion of the primary wing, and of the anterior, posterior, and distal parts of the secondary wing (Fig. 1).

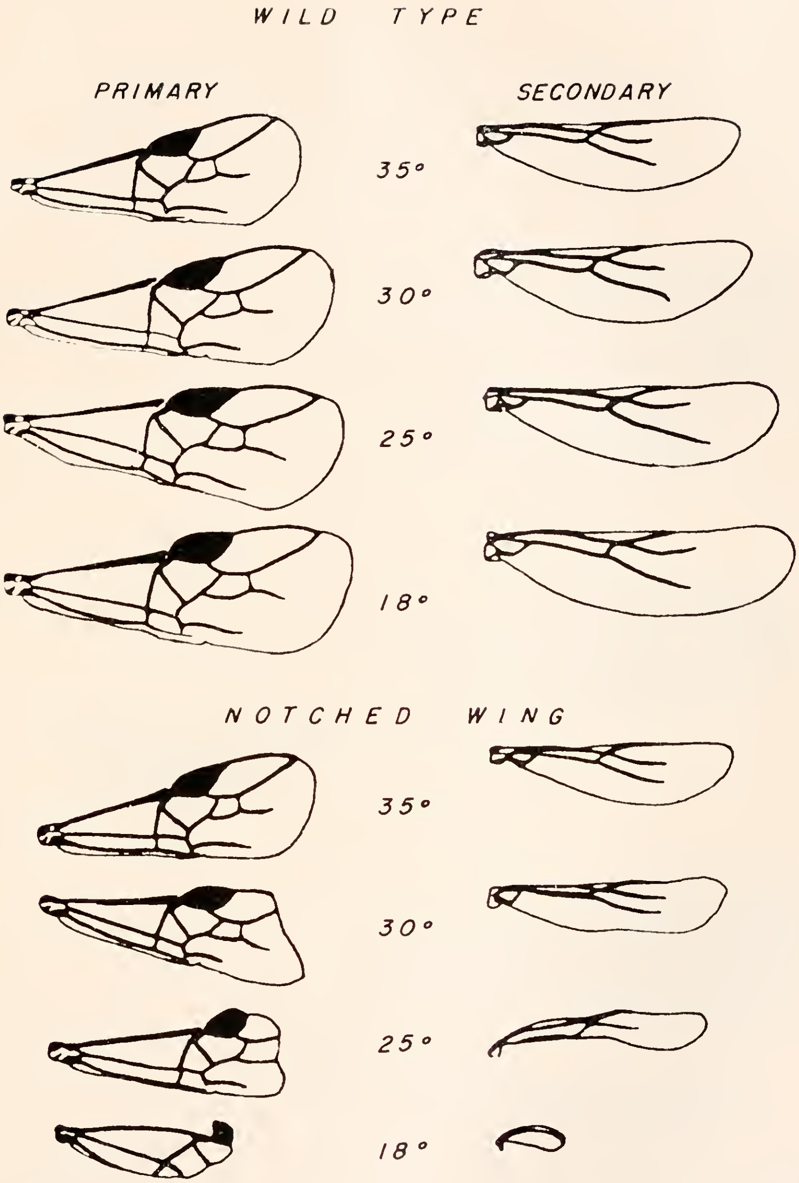
WILD TYPE
PRIMARY
SECONDARY
35°
30°
25°
18°
NOTCHED WING
35°
30°
25°
18°

FIGURE 1. Primary and secondary wings from wild type and *notched wings* wasps reared at temperatures of 35°, 30°, 25°, and 18° C.

The *small wings* mutant has smaller primary and secondary wings than wild type (Fig. 6). In addition, wing cell size is smaller than wild type as shown by the observations that the number of microchaetae per unit area is greater in the *small wings* mutant (Clark, unpublished). Each wing microchaeta corresponds to a single wing cell. Further, the microchaetae in the mutant are shorter than in the wild type.

Two different crosses were made. *Habrobracon* females homozygous for the recessive body color mutant *ebony* (*e*) were crossed with haploid males that were either *notched wings, honey body color* (*e/e* ♀♀ × *no ho* ♂♂) or *small wings, honey body color* (*e/e* ♀♀ × *sw ho* ♂♂). *Ebony* females cause the production of genetic mosaics among their progeny. *Honey* is a recessive body color mutant that shows light brown coloration. The honey phenotype can be recognized in wings as well as in other parts of the body. From these crosses, mosaics of the zygogenetic-androgenetic type were selected for further study. These mosaics were part wild type and part *honey*. The crosses were designed so that the wing mutant would always be found with *honey*. Thus, all wings, or parts of wings, that showed the *honey* phenotype were also either *notched wings* or *small wings* in phenotype. The mutant *honey* thus served as a marker for the wing mutants, *notched wings* and *small wings,* enabling their recognition in different regions of mosaic wings.

Adult wasps were collected. The wings were removed and mounted on slides with Permount. Drawings were made by means of a Bausch and Lomb Trisimplex Projector.

TABLE I

Wing mosaicism among mosaic Habrobracon

Cross	Mosaic wasps	Wasps with wing mosaicism			Intramosaic wings
		None	Inter	Intra	
e × ho no	120 (1.00)	59 (0.49)	30 (0.25)	31 (0.26)	39/480 (0.08)
e × ho sw*	150 (1.00)	88 (0.59)	30 (0.20)	32 (0.21)	39/600 (0.065)

* Data collected by David Wartell.

RESULTS

For the ebony ♀ × *notched wings, honey* ♂ cross, 120 mosaic wasps were produced. Of these, 61 showed wing mosaicism (Table I). There were 30 mosaics ("inter") defined as having individual wings either completely wild type or completely *notched wings, honey*. A bilateral mosaicism was found in which the two wings on one side were usually of one phentoype and the two wings on the other side were of the other phenotype. Among the 14 intermosaics that were found to involve two wild type wings and two *notched wings, honey*, 13 showed the presence of the wild type wings on one side and the *notched wings, honey* on the other. There were 31 mosiacs ("intra") in which both the wild type and the *notched wings, honey* phenotypes were found in the same wing. Among the 480 wings that were examined from 120 mosaic wasps, 39 wings (0.08) were found to be intramosaic (Table I).

For the *ebony* ♀ × *small wings, honey* ♂ cross, 150 mosaics were produced. The frequency of the mosaic types for this cross is comparable to the one just reported (Table I). Among 19 intermosaics that were found to involve two of each phenotype, 17 were of the bilateral pattern.

Ebony × notched wings honey

Some examples of intermosaic wing patterns are shown in Figure 2. The mosaic 2a, which developed at 30° C, shows two right wings that are wild type and two left wings that are *honey, notched wings.* The left primary wing shows notching in the anterior-distal margin; the left secondary wing shown here is like the right secondary wing in shape and size. At this temperature of 30° C the *notched wings* phenotype is sometimes not expressed. The mosaic 2b, developed at 25° C, shows a left primary wing with scalloping of the distal part and a right secondary wing that is smaller both in length and width than the wild type secondary wing on the left. The mosaic 2c, developed at 20° C, shows a more extreme reduction

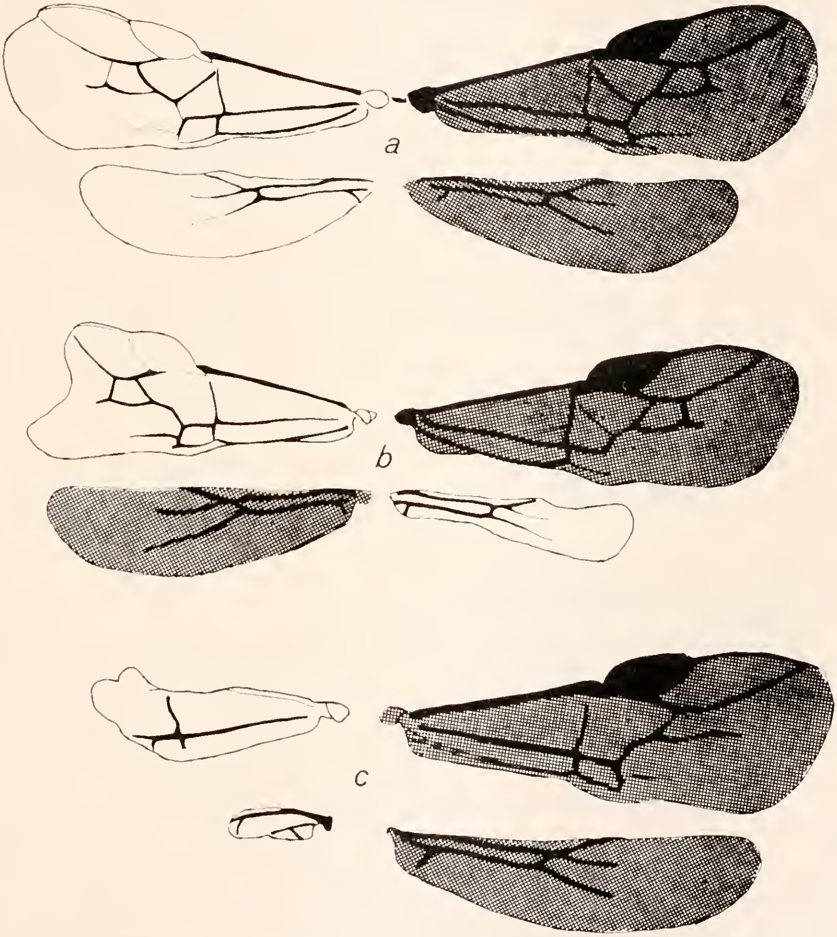

Figure 2. Wings from mosaic wasps. The crosshatched wings indicate the wild type phenotype and the clear wings indicate the *notched wings, honey* phenotype. Four wings from each of three wasps reared at 30° (a), 25° (b), and 20° (c) are shown.

in size for the *honey notched wings*. The expression of the *notched wings* phenotype at the different rearing temperatures has not been influenced by the genetic constitution of the rest of the body. There appears to be complete autonomy for the temperature sensitive mutant *notched wings*.

Mosiac wings which have a mixture of both the wild type and the *honey, notched wings* phenotype are shown in Figures 3, 4, 5. It is clear from these drawings that there is a longitudinal striping pattern (see, for example, 3a, 3f, 4c, 4i). Mor some mosaics, the stripes ran the entire length of the wing (4a, 4c, 5a); for other mosaics, the stripe ran from the middle to the distal edge (3b, 3d, 4i, 5b). A longitudinal striping pattern has been reported for wings of *Drosophila* (Bryant, 1970).

For some mosaics, two distinct phenotypic areas were observed (3b, 3c); for others, three areas were observed. In 3a, 3e, and 3f, for example, there are two separate *honey, notched wings* areas separated by a wild type area; in 4b the wild type anterior and posterior edges are separated by a *honey, notched wings* region. In terms of clonal analysis, it would seem that these separate regions of the same phenotype arose from different progenitor cells in the developing wing disc.

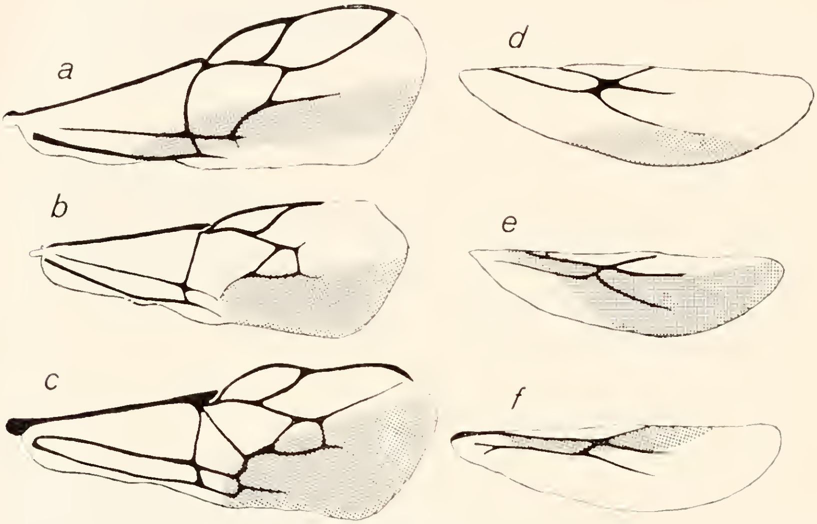

FIGURE 3. Wings mosaic for wild type (crosshatched) and *notched wings, honey,* reared at 30° C.

Different proportions of the two phenotypes may be present in each mosaic wing. In 4a, about $\frac{1}{2}$ of the mosaic wing is wild type; in 3b about $\frac{1}{4}$; and, in 3f about $\frac{1}{8}$. In *Drosophila melanogaster* the relative sizes of mosaic patches have been used to establish the number of cells that initially were set aside for the adult wing during the first few hours of embryonic development. Perhaps in *Habrobracon* the relative sizes of the two phenotypes give an indication of the relative proportions of the two different cell genotypes that go to make up the presumptive wing disc.

Mosaic wings of different shapes and sizes were obtained from wasps reared at 20° and 25° C. These shapes and sizes were determined by the amount and position of the *notched wings* genotype in the otherwise wild type wing (Figs. 4, 5). It is clear that where the *honey, notched wings* area appears, that area of the wing is smaller at the lower temperatures; and where the wild type area appears, the wing is larger. This is shown for the mosaic primary wings 5a, 5b, 5c, 5g. The mosaic wings are good examples of regional autonomy in response to temperature.

Ebony × *small wings, honey*

The four wings from a bilateral mosaic (Fig. 6a) show two left wings that are wild type and two right wings that are *small wings, honey*. The *small wings* are similar in shape, but smaller in length and width than the comparable wild type wings. For wings that are mosaic, differential growth of the wild type and mutant areas occurs. This is manifested by distortion of wing shape, wrinkles and curling of the wing. In the primary wing (Fig. 6b) where the anterior part is wild type and the posterior part is mutant, there is a downward bending of the wing. For the wings shown in Figs. 6c, 6e, 6f where the posterior part is wild type and the anterior part is mutant, there is a bending of the wing upward. The wrinkles that

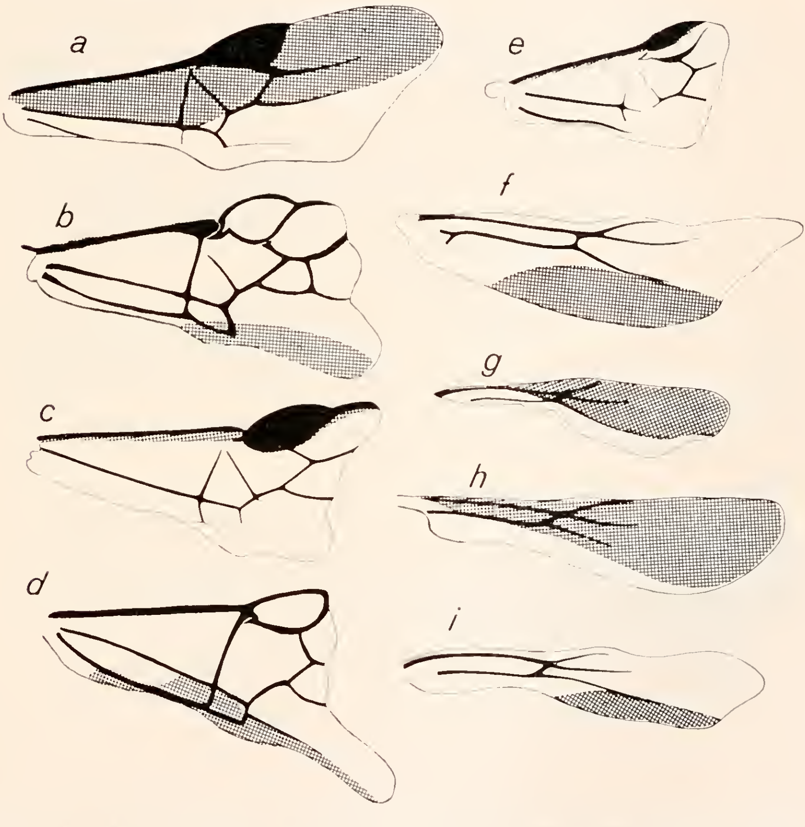

FIGURE 4. Mosaic wings for wild type and *notched wings honey*, reared at 25° C.

appeared in mosaic wings were usually in a transverse direction and occurred in the wild type region of the wing (Figs. 6c, 6d, 6g). These wrinkles are more readily seen in the secondary wings (Figs. 6d, 6g). For secondary wings, there frequently occurred a curling so that the wing had a convex and a concave surface. Attempts to flatten such wings resulted in tears at the edges or in a folding-over of part of the wing.

The wild type and *small wings, honey* areas of the mosaic wing are clearly delimited. One can recognize the longitudinal striping pattern. The *small wings, honey* region shows the shorter microchaetae, more closely spaced, lying next to the wild type region with its longer microchaetae, more sparsely spaced.

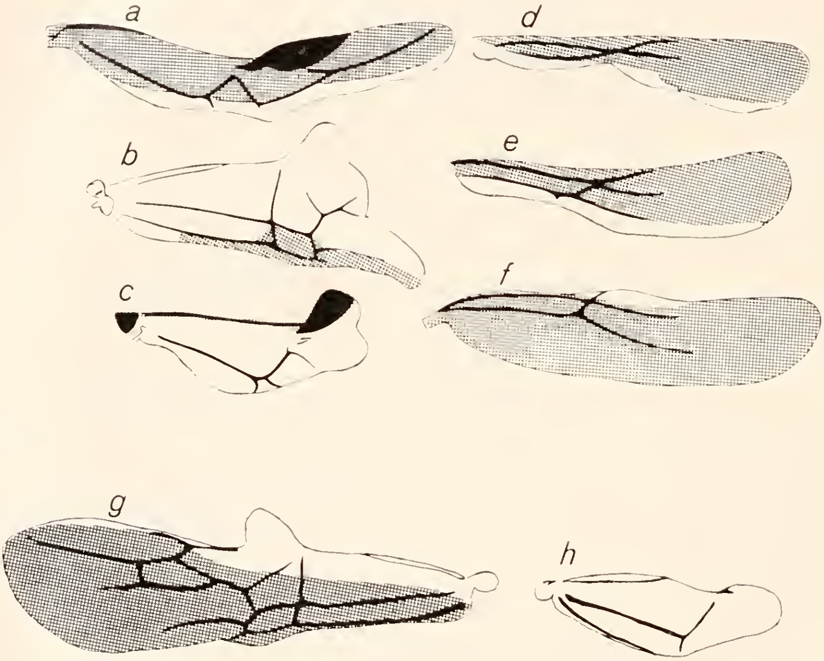

FIGURE 5. Mosaic wings for wild type and *notched wings honey,* reared at 20° C.

DISCUSSION

The wing mosaics obtained in *Drosophila* by Bryant (1970) arose as a result of X-ray-induced somatic crossing over in single cells. The size of the patches produced was related to the stage of development at which irradiation took place. Thus, in *Drosophila,* mosaicism was induced in an already formed disc or in one of a clump of cells destined to become a wing disc. Under these conditions a change in the genetic constitution of a single cell, which then proliferated, would give rise to a marked clone.

In mosaics of *Habrobracon,* nuclei of two different genotypes are already present at the start of cleavage and therefore the wing disc would already be a genetic mosaic at the time of its formation. Where the mosaic wing consisted of $\frac{1}{2}$ wild type and ½ *honey, notched wings,* the number of cells of each genetic type that went to make up the original wing disc is presumed to be equal. When ⅛ of the wing area consisted of wild type tissue, then ⅛ of the cells that made up the wing disc were wild type. Thus, one can estimate the proportion of cells, but not the number of cells, of each genotype that made up the original wing disc in *Habrobracon.*

In *Habrobracon,* some of the wing mosaics showed two longitudinal areas of the same phenotype separated by an area of different phenotype (Figs. 3a, 3e, 3f, 4b).

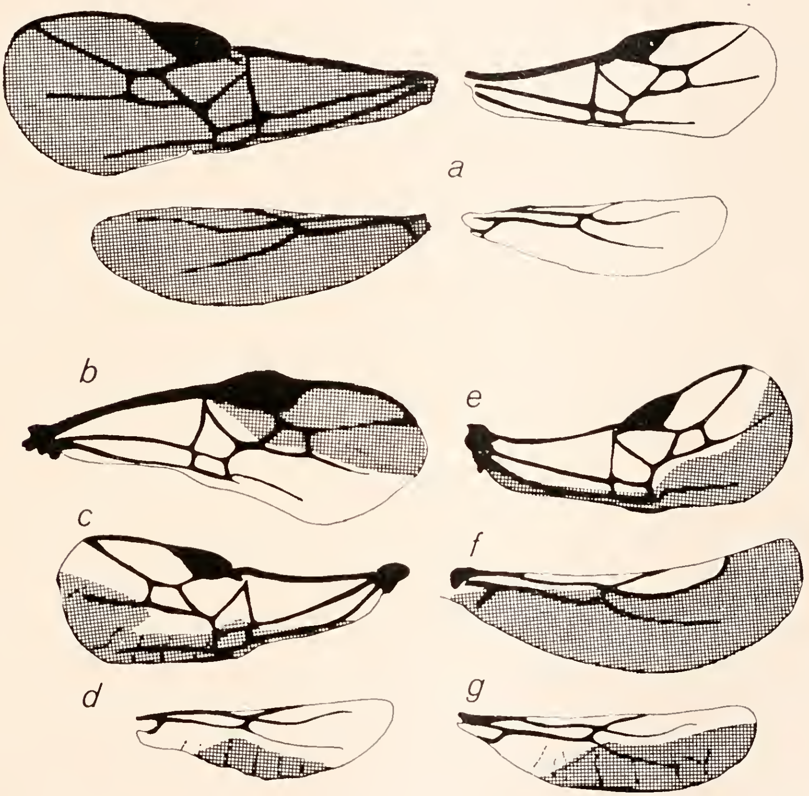

FIGURE 6. Wing mosaicism for wild type and *small wings honey,* reared at 30° C.

The explanation for this appears to be that the progenitor cells responsible for these separated regions were not contiguous in the imaginal disc. Thus, in these *Habrobracon* mosiacs one might expect to get such multiple striping patterns while in *Drosophila* somatic crossover mosaics one would not expect to get them.

The mosaic wings found in *Habrobracon* showed a longitudinal striping pattern. This type of pattern has been found for *Drosophila* wings and indicates that oriented cell divisions are important in wing morphogenesis (Bryant, 1970). This interpretation for *Drosophila* can also be used to explain the patterns of wing mosaicism in *Habrobracon.*

Under 20° and 25° C rearing conditions the proportion of wild type and *notched wings, honey* tissue in the mosaic wings of adults is probably different from that of the wing disc. The *notched wings* mutant in *Habrobracon* appears to resemble the *vestigial* mutant in Drosophila in that it is temperature sensitive, showing loss of portions of wings. In *vestigial* it has been shown that this loss is due to cellular degeneration as the wing disc grows and differentiate (Fristrom, 1968, 1969).

Mosaic wings of different shapes and sizes were determined by the amount and position of the *notched wings* phenotype. There appears to be regional autonomy not only because the *notched wings* region develops independently of wild type but also because the growth of one part of the wing is not interfered with by the other part of the wing.

For the wild type—*small wings* mosaics, changes in wing shape also were found and depended upon the relative amounts and positions of the two phenotypes. Here, however, there appears to be an influence of the growth of each part of the wing on the other. The bends, wrinkles, and curls in the mosaic wings indicate that the *small wings* and the wild type parts are growing at the same time but at different rates. The bending of the wing downward is what one might expect when the wild type part is on the anterior surface, and upward when the wild type part is on the posterior surface. The wrinkles found primarily in a transverse direction and in the wild type part of the mosaic wing are indication also that wing growth from the imaginal disc occurs at different rates in *small wings* and wild type and also that this growth occurs in a longitudinal direction.

We wish to thank Jan Hance, David Wartell, and Karen Brockmeier for their technical assistance.

SUMMARY

Habrobracon mosaics involving either wild type and *notched wings* or wild type and *small wings* were used to study mosaic patterns in wings. A longitudinal striping pattern was found for the wings of *Habrobracon*. This pattern is like that described for wings and other structures derived from imaginal discs in *Drosophila*. It indicates that cell divisions are preferentially oriented in certain directions in the developing disc (Bryant, 1970). Wings that were mosaic for *notched wings* showed regional autonomy with respect to temperature sensitivity. The different shapes and sizes of the mosaic wings were determined by the amounts and positions of the *notched wings* or *small wings* phenotype in them. Distortions in wing shape for wild type-*small wings* mosaics were related to concurrent growth and differential growth rates between the wild type and the *small wings* portions.

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THE RESPIRATORY RESPONSE TO ASPHYXIA OF *TYPHLOGOBIUS CALIFORNIENSIS* (TELEOSTEI: GOBIIDAE) AND SOME RELATED GOBIES[1]

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Fishes of the large and diverse teleost order Gobiiformes have successfully invaded many habitats characterized by rigorous and fluctuating environmental conditions. In the Californian (warm-and cool-temperate) region of the eastern Pacific are found the nine species of the *Lepidogobius* group. These gobies inhabit, with several exceptions, the shallow waters of estuaries, bays and coastal sloughs. Several species are known to take refuse in the burrow of various substrate-inhabiting invertebrates during tidal exposure, and one, *Typhlogobius californiensis* Steindachner, is an obligate commensal of the burrowing ghost shrimp *Callianassa affinis* Holmes.

Typhlogobius, the blind goby of California, is highly specialized for its unusual mode of life. The pelagic larvae have functional eyes which are overgrown by the skin soon after settling and metamorphosis. They are uncompromising in their choice of a host; adults are never found elsewhere than in *Callianassa affinis* burrows. These burrows are largely confined to the intertidal region along the open coast, in areas of mixed sand and rocks (MacGinitie, 1939).

Tolerance of hypoxic conditions would be of adaptive significance for *Typhlogobius*, since the interstitial water of marine beaches is known to often be deficient in oxygen (Gordon, 1960; Bradfield, 1964). *Callianassa affinis*, the host of *Typhlogobius*, is extremely tolerant of hypoxic conditions (Thompson and Pritchard, 1969), as are many other littoral burrowing invertebrates. Several authors have noted that *Typhologius* has a remarkable ability to withstand conditions of oxygen depletion which would be rapidly fatal to other fishes (Rosa Eigenmann, 1890; MacGinitie, 1939).

Other related gobies are found in the shallow muddy bays and sloughs of southern California. One of these, *Gillichthys mirabilis* Cooper, is known to take refuge in burrows when the tide flats are exposed, or when frightened (Barlow, 1961). The special respiratory adaptations of *Gillichthys* have been described by Todd and Ebeling (1966). *Gillichthys* is a facultative aerial breather, gulping air when conditions become unfavorable for aquatic respiration. Gaseous exchange between air bubble and blood occurs by way of the heavily vascularized epithelium of the buccopharyngeal cavity.

Another goby, *Coryphopterus nicholsii* Bean, is less closely related to *Typhlogobius* and does not belong to the *Lepidogobius* species group. In southern California it is found offshore on sand and clay bottoms from less than 10 to over 200 meters depth. Oxygen concentrations in these open waters deviate only slightly from air saturation.

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[1] From a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of California at San Diego, 1970.

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The purpose of this investigation was to compare the respiratory, metabolic, and behavioral responses of these three gobiid fishes to axyphia. Since one of these fishes (*Typhlogobius*) dwells throughout its adult life in an environment frequently impoverished with respect to free oxygen, it was expected that respiratory and metabolic adaptations of special interest might be found. Metabolic oxygen requirements were established for each of the species, and abilities to sustain oxidative metabolism at low ambient oxygen tensions were studied. Attention was given to the significance of the oxygen stores under asphyxial conditions, and also to the possible importance of anaerobic glycolysis for prolonging asphyxial survival.

Materials and Methods

All fishes were kept in individual circular specimen jars with a capacity of approximately two liters. Temperature was held at 15 ± 0.5° C and lights were cycled on a twelve hours on- twelve hours off regime by an automatic timer. Fishes were maintained under these conditions for two to eight weeks before use for experimental purposes, and were fed at intervals of two to three days on a variety of fresh and frozen foods.

Respirometry

The respirometer chamber was machined from aluminum and anodized to prevent corrosion. This chamber had a cylindrical bottom portion into which the piston-like lid was inserted, giving a closed volume of 82.9 ml. Two "O" rings on the lid insured a gas tight seal. Oxygen tension was monitored by a polarographic oxygen electrode (Beckman model 777) inserted through an O-ring seal in the lid.

The oxygen electrode was calibrated in air, at 15° C and 100% relative humidity, before every experiment. A constant rate of stirring was maintained by a magnetic stirring bar. Because the bar would not spin reliably beyond a certain rate, it was necessary to stir at a suboptimal rate and apply a corrective factor to the calibration (Cary and Teal, 1965). The calibration correction was determined by calibrating the electrode in air and checking the electrode readout in sea water against the conventional Winkler method over a wide range of oxygen tensions. Once calibrated, the oxygen electrode was quite stable; electrode drift never exceeded 2% of full scale after 24 hours of operation.

The change in oxygen tension in the respirometer chamber was monitored by the oxygen analyzer as it was progressively lowered by respiration of the experimental animal. Changing oxygen tension was recorded on a 5 inch chart recorder (Bausch and Lomb VOM-4). These data, along with the volume of the chamber, the solubility of oxygen in seawater, and the weight of the animal, allowed the rate of oxygen uptake per unit weight of animal to be calculated over any chosen oxygen tension interval. All respirometry was carried out at a temperature of 15 ± 1° C and a salinity of approximately 33.6‰.

Animals were starved for several days before experimental runs so that they would be in a postabsorptive state. Microbial respiration was eliminated by filtering all seawater through a 0.4 μ glass filter (Millipore) and by adding 100 mg of streptomycin sulfate per liter.

Because excitement and fright of fishes due to handling is known to cause increased activity and an elevated rate of oxygen consumption (Wells, 1932; Fry, 1957), the fish used in this study were protected from outside stimulation in several ways. The interior of the respirometer was darkened, and the respirometer was acoustically insulated by placing a ½ inch felt mat beneath it. From 8 to 12 hours were allowed for diminution of handling excitement and for adjustment to the chamber. A relatively constant rate of oxygen uptake was observed in most cases after this period of time. A flow of aerated, temperature equilibrated seawater was maintained through the respirometer throughout the acclimation period.

Elevated oxygen consumption rates were sometimes noted and were associated with high levels of activity. Although it was not possible to observe fishes in the respirometer, an estimate of activity could be obtained by taking advantage of the sensitivity of the oxygen electrode to stirring. The magnetic stirring motor was switched off and the drop in oxygen tension noted. At very low levels of activity, agitation of the water in the respirometer due to movements of the fish was minimal and the mean indicated oxygen tension would drop to about 50% of the true value. At high levels of activity, the mean indicated oxygen tension would drop only 15–30%, showing many sharp upswings corresponding to individual swimming movements.

Gasbladder sampling

Samples were removed from the gasbladder by body wall puncture and withdrawn into a 1 ml acid-citrate filled syringe. The fish were anesthetized with quinaldine (Eastman Kodak) and restrained underwater during sampling to prevent accidental contamination with air. A portion of the gas sample was transferred to a Scholander microgas analyzer (Scholander, Van Dam, Claff and Kanwisher, 1955) for determination of oxygen, carbon dioxide, and nitrogen (inert gas) content. Duplicate analyses generally agreed within 1 vol% or better.

Reliable estimates of gasbladder volume in intact fish were obtained by pressure-volume manometry (Kanwisher and Ebeling, 1957). Five or six replicate measurements of volume were made for each fish and averaged; replicates usually differed by less than 10%. Blank compliance of the apparatus was approximately 3 μl, so calculated gasbladder volumes were corrected by this volume.

To determine whether anoxic individuals of each of the three species could metabolize gasbladder oxygen, fishes were asphyxiated in sealed syringes for various periods of time before their gasbladders were sampled. Fishes confined in unsealed syringe barrels served as controls, except for several controls which were taken directly from aerated water.

Lactate production and excretion

Asphyxiated fishes were assayed for lactic acid to determine whether energy was being derived from anaerobic glycolysis. Fishes were weighed and 24–48 hours later sealed in flasks or syringes filled with nitrogen-equilibrated seawater.

After a predetermined period of time they were removed, decapitated, and rapidly ground in a mortar with 6 to 24 body volumes of 0.6 M perchloric acid. The ground tissue and perchloric acid were allowed to equilibrate for 1 to 2 hours with occasional stirring; equilibrium was attained in less than 1 hour. After centrifugation the supernatant was assayed for lactate enzymatically, using commercial (Calbiochem or Biochemica) reagents; duplicate analyses using both sets of reagents agreed within 3 mg%. The test reagents contained nicotinamide-adenine dinucleotide (NAD) and lactate dehydrogenase, which reacted with lactate to produce pyruvate and reduced NAD (NADH). The reaction was driven to completion by trapping of pyruvate. Absorption of the NADH formed was measured at 340 mu with a Bausch and Lomb (Spectronic 20) spectrophotometer. Two or more lactate standards of known concentration were run with each set of samples so that net absorptions could be accurately converted to lactate concentrations. Samples were usually determined in duplicate and averaged.

The possibility that *Typhlogobius* might excrete lactate ion either during or following asphyxia was also investigated. Water samples were taken from syringes in which specimens of *Typhlogobius* had been confined with 7 to 10 ml of nitrogen-equilibrated seawater for various periods of time. Samples were also taken from open beakers of aerated seawater in which individuals had been allowed to recover after long periods of asphyxia. All water samples were mixed 1:1 with perchloric acid and assayed for lactate as previously described. Blanks and standards were prepared using seawater.

Asphyxial survival

Survival ability under asphyxial conditions was studied by observing fishes sealed in syringes or flasks of deoxygenated seawater. Deoxygenated seawater was prepared by two different procedures. The first involved heating several liters of seawater to boiling and then cooling rapidly while bubbling with nitrogen. This treatment lowered the oxygen content to nearly zero, and also raised the pH to approximately 8.7 by removing dissolved CO_2. The second procedure was to seal several *Gillichthys* in a flask of seawater for 12–20 hours. Respiration of the confined fishes lowered the oxygen tension of the water to 3 mm Hg or less, and the pH to 7.2–7.3.

Both glass syringes and glass-stoppered flasks of various sizes were used in survival experiments. When flasks were used, they were first inverted in a water bath and the contained water displaced with nitrogen. The nitrogen was then displaced with deoxygenated water. After filling and overflowing the flask, the experimental animal was introduced and the flask sealed. Contamination of syringe water was minimized by flushing with 5–6 volumes of deoxygenated water after placing the animal inside. Oxygen contamination resulting from these procedures was shown by repeated blank determinations to be less than 3 mm Hg.

After an experiment was begun, fishes were observed at frequent intervals. The end point chosen was total cessation of respiratory movements. When inverting the flask or syringe no longer elicited even feeble brachiostegal movements, the animal was removed. Often a water sample was taken from each flask or syringe for determination of final pH (Corning Model 12 pH meter).

Burrow habitat sampling

In order to determine the pH and PO_2 prevailing in the burrows of *Callianassa* during tidal exposure, a piece of polyvinylchloride tubing was pushed into the burrows and water samples were withdrawn into a 10 ml glass syringe. Contamination was avoided by use of a three way syringe stopcock which allowed the dead space to be cleared of air and flushed before filling of the sample syringe. Interstitial water samples were obtained by a sampling device constructed from stainless steel tubing, which was pushed into the substrate to the desired sampling depth.

Sample syringes were sealed with a gastight plug and stored on ice until they could be returned to the laboratory and analyzed, usually less than five hours after collection. Sample oxygen tensions were determined in the laboratory with an oxygen electrode (Radiometer) calibrated against N_2 and air. The absence of significant oxygen tension changes in the iced syringes was confirmed by comparing the oxygen tensions of replicate samples taken from the syringes at intervals of several hours.

RESULTS

Relation between oxygen tension and oxygen uptake rate

Quiescent individuals of each of the three species regulated oxygen uptake over a wide range of oxygen tensions. The four typical plots shown in Figure 1 indicate that oxygen uptake began to decline only after the oxygen tension had fallen to a relatively low level.

In about 20% of the *Typhlogobius* respirometric experiments there was evidence of persistent activity even after 8 to 12 hours of acclimation to the respirometer chamber. The level of activity was frequently estimated by observing indicated oxygen tension readings in the absence of stirring. Active animals produced many rapid fluctuations in the indicated reading, corresponding to individual swimming movements. The concomitant oxygen uptake data invariably indicated a high and fluctuating uptake rate, which usually declined as the oxygen tension in the chamber decreased. Such an "oxygen-dependent" respiratory pattern has been described in various fish species, and Beamish (1964a, 1964b) has demonstrated a correlation between oxygen dependence and muscular activity in several species of fish. In the present study, data showing a strongly fluctuating or steeply declining oxygen uptake rate due to activity were discarded.

In order to characterize the tension range associated with loss of ability to regulate resting oxygen uptake, it was determined within which 2% saturation interval uptake first fell to 85% or less of the resting rate determined for that individual fish. The *preceding* (higher) 2% interval was designated as the critical oxygen tension interval. The 85% definition was arbitrarily chosen, but the break in slope was usually sharp, and an 80% or 90% criterion would have had little effect on the critical levels established for each of the species. The critical interval for each plot in Figure 1 is indicated by an arrow.

Critical tensions determined for individuals of a species fell within a limited range, as shown in Table I. Disregarding the single highest value for each species, the range of critical oxygen tensions was 6–10% saturation (9–16 mm Hg) for *Typhlogobius*, 10–16% saturation (16–25 mm Hg) for *Gillichthys*, and 12–18% (19–28

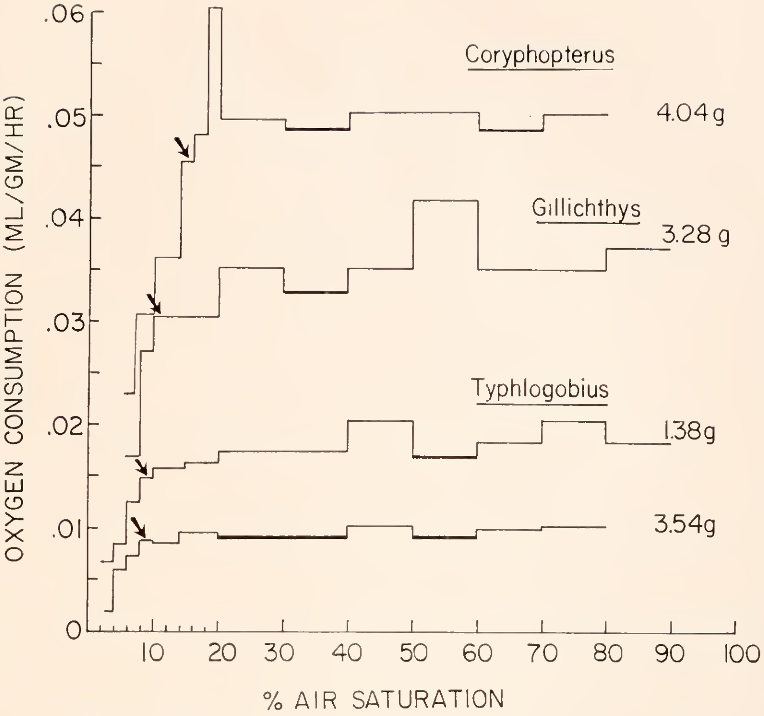

FIGURE 1. Four typical plots showing oxygen consumption at 15° C as a function of oxygen tension. The heavy lines indicate resting oxygen consumption rates and the arrows designate the tension at which oxygen consumption becomes tension-dependent (critical level). Wet body weights are given for each example.

mm Hg) for *Coryphopterus*. Critical oxygen tension distributions were compared by the Neuman-Keuls analysis of variance (Sokal and Rohlf, 1969) and found to differ significantly at the 0.01 probability level in all comparisons.

The Kruskal-Wallis sum of ranks test (Tate and Clelland, 1957) was used to test for possible correlation between weight and critical interval within species. No correlation was indicated ($P > 0.1$ in each case).

TABLE I

Distribution of critical oxygen tension intervals (1% saturation = 1.56 mm Hg).

	N	Critical intervals (% Saturation)						
		6–8%	8–10%	10–12%	12–14%	14–16%	16–18%	18–20%
Typhlogobisu	13	4	8	1				
Gillichthys	15			6	5	3	1	
Coryphopterus	11				4	3	3	1

Resting oxygen consumption

For estimation of the resting or "standard" metabolic rate, the rate of oxygen uptake was calculated for each 10% saturation interval down to and including the 40–30% interval (which was safely above the range where oxygen uptake became tension dependent and began to decline). The lowest uptake rate calculated for any 10% saturation interval was taken as the resting rate. This procedure is similar to that followed by Poulson (1963) and Job (1955) who made serial determinations of oxygen uptake after a predetermined calming period, and recorded the lowest as the standard metabolic rate. The presumed resting (standard) oxygen consumption rate is indicated by a heavy line in each of the four examples of Figure 1.

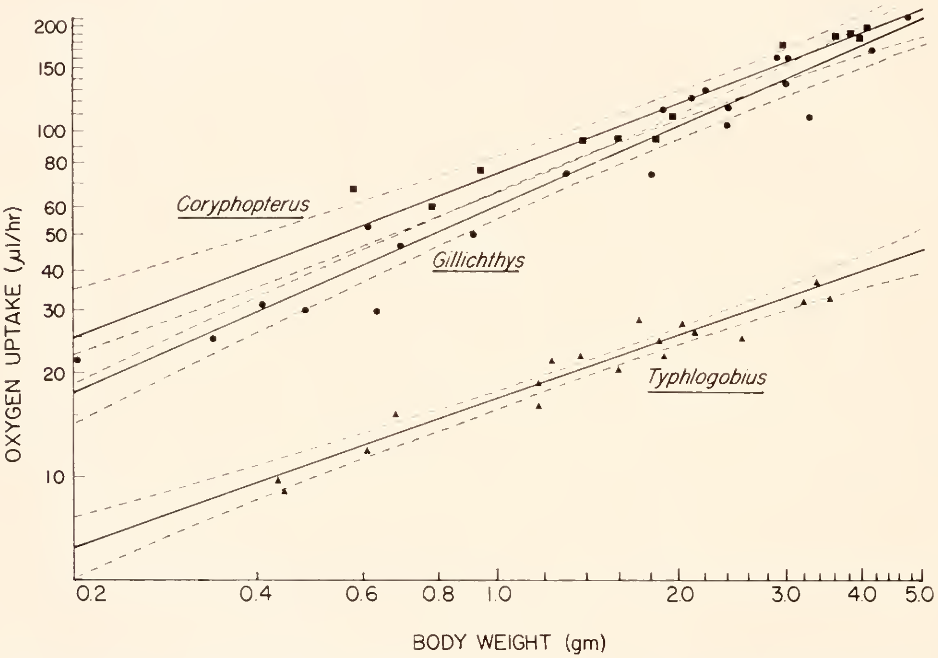

FIGURE 2. The relation between log wet body weight and log resting oxygen consumption for *Typhlogobius*, *Gillichthys*, and *Coryphopterus*, with 95% confidence limits.

Relation between weight and resting oxygen consumption

Regression of the logarithm of resting oxygen consumption per total weight on the logarithm of wet weight was calculated by the Bartlett (1949) procedure (Fig. 2). Linearity tests confirmed a good fit to a straight line for each of the three species. Regressions of log uptake/unit weight on log wet weight tended to be curvilinear (Marr, 1955).

In every case regression coefficients were less than one, indicating that resting oxygen consumption was not linearly related to wet body weight (Table II). Comparison of slopes by a modification of the Scheffe multiple comparison test (Scheffe, 1959) indicated there were no signficant differences. However, the regressions

TABLE II

The relation between resting oxygen uptake and wet body weight. The regression equation $\log M = \log a + b \log Wt$; *M is* $\mu l\ O_2/hr$ *and Wt is grams*

	N	a	95% C.L. for a	b	95% C.L. for b
Typhlogobius	18	16.6	15.7–17.4	0.615	0.521–0.716
Gillichthys	20	59.0	54.1–64.3	0.762	0.657–0.871
Coryphopterus	13	74.0	68.4–79.8	0.676	0.551–0.825

did differ significantly in position as indicated by a comparison of the 95% confidence limits for mean predicted oxygen uptake in Figure 2. It is evident that *Typhlogobius* consumes oxygen at a much lower rate than either of the other two species.

Utilization of gasbladder oxygen

Gasbladder volumes determined by volume-pressure manometry for six individuals of each species and expressed as ml gas/100 g were as follows ($\bar{x} \pm$ S.D.): *Typhlogobius*, 3.9 ± 0.2; *Gillichthys*, 3.2 ± 0.9; and *Coryphopterus*, 3.5 ± 1.06. Volumes of *Typhlogobius* gasbladders ranged from 3.6 to 4.1 ml/100 g. Data for the two other species were more variable; high values were close to or within the *Typhlogobius* volume range, but several individuals apparently had partially deflated gasbladders.

The composition of gases in the gasbladder was also quite variable (Table III). *Gillichthys* gasbladder oxygen ranged from 3.4 to 53 vol%, even though all animals had been resting quietly in well aerated seawater for at least 10 days prior to sampling.

Both *Typhlogobius* and *Gillichthys* utilized gasbladder oxygen rapidly under asphyxial conditions (Table IV). Oxygen in the gasbladder of *Typhlogobius* had declined to less than 2 vol% after 2 hours of asphyxia and to less than 1 vol% after 4 hours. *Gillichthys* gasbladder oxygen had declined to 3 vol% or less after 1.5 to 2.5 hours of asphyxia. The limited accuracy of the analytical procedure made it impossible to determine whether gasbladder oxygen was evenutally completely utilized.

Typhlogobius gasbladders sampled in the field were not depleted of oxygen. Five individuals were collected on a −2.2 foot low tide in January, 1969. They

TABLE III

Gasbladder O_2 *and* CO_2

	N	O_2 (vol %)		CO_2 (vol %)	
		$\bar{X} \pm$ S.D.	Range	$\bar{X} \pm$ S.D.	Range
Typhlogobius	7	29.3 ± 18	12–67%	0.6 ± 0.4	0–1.0%
Gillichthys	18	24.8 ± 17	3–53%	1.1 ± 0.7	0–2.6%
Coryphopterus	7	34.1 ± 17	20–51%	1.2 ± 0.9	0–2.8%

were sampled at intervals of 20–30 minutes, beginning one hour before low water, and ending one hour after. The percentage oxygen in the gasbladders of these fishes ranged from 18.1% to 27.4% (24.6%, 18.1%, 27.4%, 22.2%, 25.4%) with a mean of 23.5%, agreeing reasonably well with the mean 26.4% oxygen found in gasbladders of 11 laboratory acclimated animals (Tables III and IV).

The mean oxygen in gasbladders of four specimens of *Coryphopterus* which succumbed to asphyxia was somewhat lower (23.8 ± 14 vol%) than mean oxygen in gasbladders of aerobic controls (34.1 ± 15 vol%), but the difference was not significant ($P < 0.20$; Mann-Whitney U test). It is apparent that *Coryphopterus* does not use the gasbladder as an emergency oxygen store to any important degree.

Lactate production and excretion

Accumulated lactate was expressed as μM per gram of wet weight. Since fishes of differing weights were used in these experiments, the data were corrected for the effect of weight on metabolic rate. It was assumed that the relation established between weight and aerobic metabolism rate ($M = aWt$, Table II) could be extended to describe the relation between weight and anaerobic metabolic rate. Correction was made to the mean weight of all fish of a species used in this experiment ($\bar{x}$ weight *Typhlogobius* = 1.5 g; $\bar{x}$ weight *Gillichthys* = 2.5 g). The lactate concentration found in an x gram *Typhlogobius* was multiplied by the corrective factor M/Wt (1.5 g fish)/M/Wt (x g fish); M is the calculated resting total oxygen uptake (Table II). This correction altered most lactate values by less than 15%.

A linear regression equation of corrected *Typhlogobius* lactate values *versus* duration of asphyxia was fitted by the least squares procedure. The significant fit (linearity t test) of the data indicated a steady rate of lactate accumulation up to and presumably beyond the forty-sixth hour. The regression slope was 1.16 μM/g/hr with 95% confidence limits of 0.90 to 1.41 μM/g/hr.

The *Gillichthys* lactate accumulation data were much more variable than the *Typhlogobius* data, perhaps as a result of the higher metabolic rate and more active behavior. The least squares regression slope was 3.3 μM/g/hr with broad 95% confidence limits overlapping zero (−0.96 to 6.9 μM/g/hr).

Table IV

Utilization of gasbladder oxygen under asphyxial conditions

Typhlogobius			Gillichthys		
Time	n	% O_2 remaining	Time	n	% O_2 remaining
Control	4	21.4	Control	4	35.0
1 hr	1	4.4	10 m	1	43.0
2 hr	1	1.3	20 m	2	20.2
3 hr	1	1.0	30 m	1	14.0
4 hr	1	0.9	1 hr	3	11.0
6 hr	1	0.3	1 hr 30 m	2	2.2
21.5 hr	1	0.3	2 hr	2	1.7
25.5 hr	1	0.0	2 hr 30 m	1	0.3

There was no indication that *Typhlogobius* could excrete lactate ion either during asphyxia or recovery from asphyxia. None of the seawater samples assayed for lactate deviated from zero by more than ± 0.3 μM/l, approximating the limits of precision for the analytical procedure.

Asphyxial survival

Specimens of *Typhlogobius* and *Gillichthys* initially responded to deoxygenated water by increasing ventilation rate and volume, but this phase was brief and soon followed by a pronounced slowing or complete cessation of ventilation. Eventually both species resumed respiratory movements at a low amplitude and rate. Respiratory movements gradually became increasingly irregular and infrequent and finally ceased entirely.

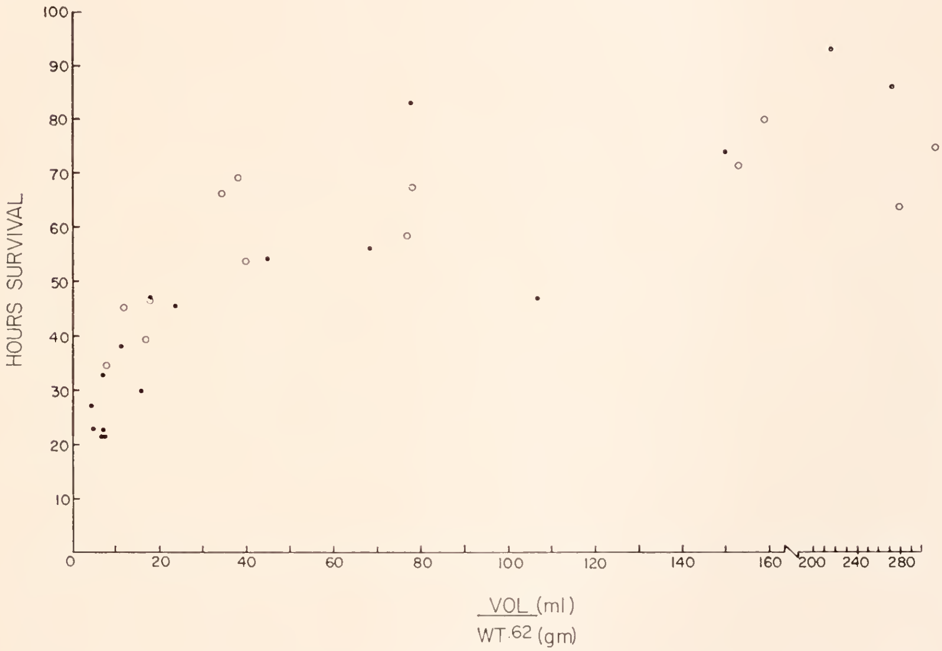

FIGURE 3. Relation between asphyxial survival of *Typhlogobius* and seawater volume/wet body weight ratio (corrected for metabolic rate). Closed circles indicate an initial seawater pH of 7.2 to 7.4; open circles an initial pH of 8.7. The initial P_{O_2} was always less than 3 mm Hg.

Coryphopterus responded quite differently to deoxygenated water. The initial ventilatory response was a series of convulsive "coughs." The fish then began to swim violently upward, continuing until exhaustion curtailed further effort.

The time taken for respiratory movements of asphyxiated specimens of *Typhlogobius* to fail ("survival time") was found to vary with the volume of enclosing seawater. This is shown in Figure 3 by the plotting of survival time against the ratio seawater volume(ml)/wet body weight(g)$^{0.62}$. The 0.62 power of the body weight was used since it had been determined by respirometry to bear a proportional relationship to metabolic rate (Table II), and it was felt that asphyxial survival time should be more closely correlated with the inverse of metabolic rate than with the inverse of body weight. The ratio seawater volume (ml)/wet body weight (g)$^{0.62}$ will be referred to as "relative volume." The data points of Figure 7 may be fitted by an exponential regression equation of the form $x = pe^{ky}$. Rewritten with y (hours survival) as the dependent variable, and replacing x (volume/Wt$^{0.62}$) with logarithm x, the relationship becomes linear: $y = -0.796 + 14.946 \ln x$ (Bartlett regression). A linearity t test indicated that this regression provides a good fit to the data points. The 95% confidence intervals were ±2.94 for the slope and ±3.53 for the y intercept.

The survival of asphyxiated specimens of *Gillichthys* was independent of the relative volume of enclosing seawater. Survival times ranged from 6.5 to 12.5 hours for well nourished animals acclimated for one month. Another group which had been kept in the laboratory for four to five months and fed at irregular intervals survived only 1.5 to 4.5 hours of asphyxia.

Coryphopterus survival also was independent of volume. Survival times in nitrogen-equilibrated seawater ranged from 28 to 42 minutes.

Although cessation of ventilation was selected as a convenient end point for survival experiments, the animals were not necessarily dead when ventilation ceased. *Typhlogobius* and *Gillichthys* could be revived by removal to fresh seawater after no signs of consciousness or life had been apparent for some time. *Typhlogobius* often recovered after several hours in this state, although recovery following long periods of asphyxia was quite protracted. Often animals in the eighth hour of recovery or later would still not have regained equilibrium, and would be hyperventilating rapidly. However, no lasting effects could be detected. Animals revived after 80–90 hours of asphyxia took food the following day and lived for months afterwards in the laboratory.

In contrast, irreversible changes apparently occurred in the central nervous system of *Coryphopterus* a few minutes following stoppage of respiration. These changes were made manifest initially by twitching or quivering of the gill arches and pectoral fins. Shortly afterwards the dorsal and anal fins began to quiver and occasionally the trunk muscles would perk sharply. These convulsive movements lasted for 15–20 minutes, and the animals could not be revived once they had begun.

The initial carbon dioxide content of deoxygenated seawater did not seem to have any effect on the survival of *Typhlogobius.* The open circles in Figure 3 represent survival of animals in nitrogen-equilibrated seawater with an initial pH of 8.7; closed circles represent survival of animals in *Gillichthys*-preconditioned seawater with an initial pH of 7.2–7.3.

The pH of seawater contained in syringes and flasks was lowered by the metabolism of enclosed specimens of *Typhlogobius.* The terminal pH at cessation of respiration was related to the initial pH and to the relative volume of seawater. At lower relative volumes (< 10) a minimum pH of approximately 5.4 was attained, regardless of the initial pH. The inflection point in the asphyxial survival curve (Fig. 3) at a relative volume of approximately 25–35 coincides with a terminal pH of approximately 5.7–5.8; this relationship is more clearly shown by Figure 4.

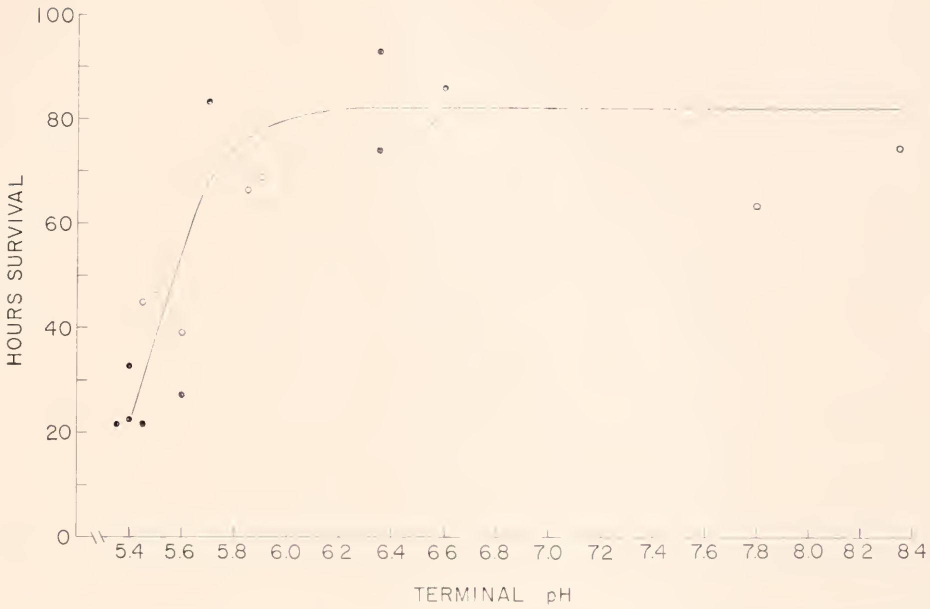

FIGURE 4. Correspondence between survival time of asphyxiated *Typhlogobius* and terminal seawater pH. Closed circles indicate an initial pH of 7.2–7.4; open circles an initial pH of 8.7

The decrease in seawater pH was in part due to excretion of CO_2. However, because of the limited amount of oxygen available in the gasbladder, only a small amount of CO_2 could have been produced by oxidative metabolism. With a respiratory quotient of unity, the oxygen contained in the gasbladder of a 1.5 g *Typhlogobius* (4% body volume; 25 vol% O_2) could produce about 0.7×10^{-3} mM of CO_2. Additional CO_2 would have been produced by bicarbonate buffering of anaerobically produced lactic and pyruvic acids. If *Typhlogobius* has large bicarbonate reserves in the blood comparable to those of the electric eel *Electrophorus* (30 mM/l; Johansen, Lenfant, Schmidt-Nielsen and Petersen, 1968), and if 5% of the body weight is blood, the blood would contain 2.3×10^{-3} mM bicarbonate that could be converted to CO_2. The total amount of metabolic CO_2 and buffer-produced CO_2 (3×10^{-3} mM) would lower the pH of 20 ml of seawater by about 0.4 pH units (Strickland and Parsons, 1965). A 1.5 g *Typhlogobius* confined in 20 ml of seawater (relative volume = 16) actually lowered the pH by 2–3 units.

Additional evidence indicated that carbon dioxide was not the only acid substance excreted by *Typhlogobius* during asphyxia. The presence of a non-volatile acid was shown by the observation that asphyxial seawater could not be returned to the pH of normal air-equilibrated seawater (8.0–8.2) by bubbling with air. Aeration of seawater with a terminal pH of 5.4 raised the pH to approximately 7.5. No further change in pH occurred after the first hour of aeration, even when bubbling was continued for eight hours or more. Control trials with CO_2-saturated water indicated that one-half hour of aeration was sufficient to wash out all dissolved CO_2.

It may be hypothesized that an acid byproduct of anaerobic metabolism would, if excreted at a constant rate, attain a concentration proportional to the survival

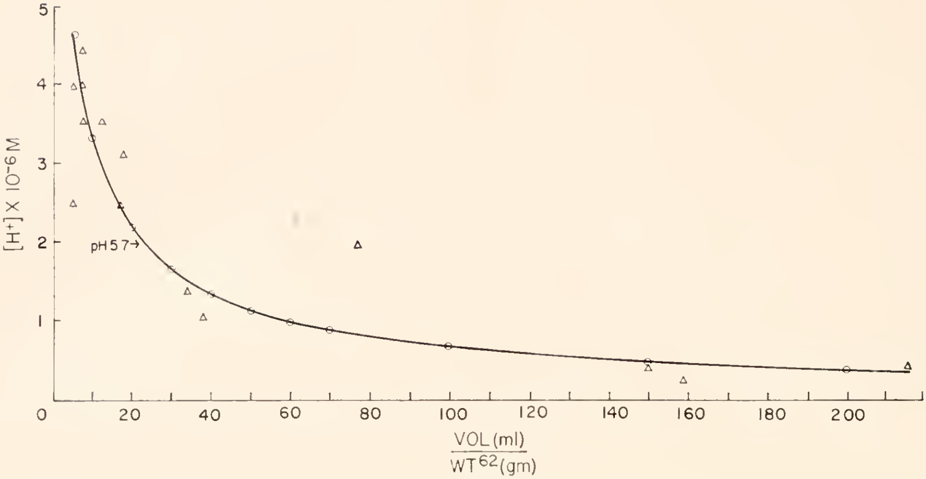

FIGURE 5. Terminal concentration of excreted metabolic byproducts following asphyxiation of *Typhlogobius* in different volumes of seawater. Circles represent the calculated concentration (arbitrary units) of a byproduct excreted at a constant rate throughout the period of asphyxiation. Triangles represent actual measured hydrogen ion concentrations.

time and inversely proportional to the relative volume ($\mathrm{Vol}/\mathrm{Wt}^{0.62}$). An equation relating survival time to relative volume has previously been derived, so the terminal byproduct concentration may be expressed:

$$\mathrm{Conc.} = \mathrm{k}\,\frac{-0.796 + 14.95(\mathrm{Vol}/\mathrm{Wt}^{0.62})}{\mathrm{Vol}/\mathrm{Wt}^{0.62}}$$

where k is a constant. If this expression is plotted in arbitrary units against relative volume (represented by the plotted curve in Figure 5) it may be seen that the estimated byproduct concentration begins to increase rapidly below a relative volume of about 25 to 35. Plotting of actual measured hydrogen ion concentration (terminal pH) against relative volume in Figure 5 shows close agreement with this expression; hydrogen ion concentration is seen to increase rapidly below a relative volume of 25 to 35, corresponding to a pH of 5.7 to 5.8. Figures 3 and 4 indicate that survival time falls off rapidly below relative volumes of 25 to 35 and below a pH of 5.7 to 5.8. This suggests that the exponential decrease in asphyxial survival time of *Typhlogobius* with decreasing seawater volume may result from accumulation of an excreted acid metabolite.

Sampling of burrow habitat

The area selected for sampling of *Callianassa affinis* burrow water and interstitial water was a shingle and sand beach at Point Loma, California. Most individuals of *Typhlogobius californiensis* used in this study were collected in the immediate area. The sampling site was at an elevation of 25 to 30 cm above Mean Lower Low Water and remained exposed for 5.5 hours on a −1.5 foot low tide.

Six interstitial water samples were taken at 15 minute intervals from a depth of 20 cm in the substrate while the sampling area was still 5 to 25 cm awash. Oxygen tensions were 3.0, 0.8, 3.8, 6.1, and 3.5 mm Hg. A transient increase in interstitial oxygen tension was observed as the surface drained; this may have been caused by a downward percolation of surface water. Successive samples showed a rapid decline in oxygen tension. In two hours the oxygen tension fell to 4 mm Hg, followed by a further slow decrease to about 1 mm Hg after four hours.

The oxygen tension of water from burrows of *Callianassa* differed little from the oxygen tension of interstitial water, declining at the same rate to the same low levels. Four hours after tidal exposure samples from a depth of 15 to 18 cm in two burrows of *Callianassa* had oxygen tensions of 0.5 and 3 mm Hg.

DISCUSSION

Many burrowing invertebrate animals are suspension feeders, creating a current through the burrow which carries in particles of food from the outside. *Callianassa affinis,* the shrimp that serves as host for *Typhlogobius californiensis,* causes a feeding current to flow through its burrow by the action of the abdominal swimmerets (MacGinitie, 1939). The entrances to *Callianassa affinis* burrows are submerged for a major portion of every day (18 to 24 hours), and the frequently renewed water in the burorws remain relatively well oxygenated during this time. In the interstitial water outside the burrows low oxygen tensions and pHs generally prevail, due to the limited rate of exchange with the overlying oxygen-rich water.

Low minus tides may expose the burrows of *Callianassa* for 5 to 6 hours. Circulation of water through the burrows is interrupted during the period of exposure, and oxygen tensions in the deeper portions rapidly equilibrate with the interstitial water, falling to almost anoxic levels. However, some diffusive exchange with the air can occur at the burrow entrance, the rate of exchange being severely limited by the small interface area. Farley and Case (1968) have shown that *C. affinis* responds to low oxygen tensions with an increase in ventilatory rate. Under natural conditions acceleration of ventilatory activity would mix oxygenated water deeper into the burrow and enhance the rate of invasion of oxygen.

A marked tendency for specimens of *Typhlogobius* to move into a flow of hypoxic water was observed in laboratory experiments (unpublished observations). In the field this response to hypoxia should result in the goby moving upward in the burrow to a position near its hyperventilating host *Callianassa.* The water in the upper part of the burrow need be only partially oxygenated (9–12 mm Hg) for *Typhlogobius* to satisfy its meager oxygen requirements.

The fact that none of the *Typhlogobius* gasbladders sampled in the field were depleted of oxygen also argues that the fish ordinarily avoid asphyxial conditions. Induced asphyxia in the laboratory resulted in almost complete utilization of gasbladder oxygen within 1 to 2 hours.

Callianassa affinis burrows could not be expected to always remain open to the surface. The habitat is exposed to the open sea, and even the large rocks which ordinarily shelter the burrows are sometimes shifted about by the action if unusually powerful waves. At such times many burrow entrances must be blocked or filled in. If denied access to the surface, *Typhlogobius* would be unable to escape the hypoxic conditions prevailing in the substrate, and would have to rely largely upon anaerobic metabolism until the host shrimp could open a new entrance. Because the reproductive potential of these long-lived fishes is spread over a period of many years (MacGinitie, 1939), such circumstances could occur infrequently and still be strongly selective for anaerobic competence.

The relations established between body weight and oxygen consumption for the species studied are comparable to those found for other species by other investigators. The regression of log oxygen consumption against log weight generally is linear with a slope between 0.70 and 0.90 (*cf.* Fry, 1957; Beamish, 1964a). The log rate-log weight regression slope of 0.615 found for *Typhlogobius* is somewhat lower than most reported slope values, and may be of adaptive advantage in reducing the oxygen requirements of larger individuals.

Typhlogobius has a very low rate of resting oxygen consumption, only 20–30% that of *Gillichthys* and *Coryphopterus*. *Typhlogobius* oxygen requirements are also much smaller than most oxygen uptake rates reported in the literature for inactive fishes (Beamish, 1964b). The depressed metabolic rate of *Typhlogobius* may serve to bring food and oxygen requirements into balance with limited supplies available in the habitat. A low metabolic level would also conserve energy substrate stores during periods of protracted anaerobiosis.

Typhlogobius, Gillichthys and Coryphopterus regulate oxygen consumption down to oxygen tensions of 9–16, 16–25, and 19–28 mm Hg, respectively, at 15° C. The critical oxygen tensions found for these gobies support the generalization that ability to regulate oxygen consumption is correlated with oxygen availability in the habitat; animals living in periodically or chronically stagnant waters, such as *Typhlogobius,* usually have lower critical oxygen tensions than those living in well aerated waters (Prosser, 1955). However, even *Coryphopterus* regulates to a relatively low tension in comparison with many other fishes. It is interesting that the critical oxygen tension range found for *Typhlogobius* (9–16 mm Hg) is in close agreement with the critical oxygen tension range established for *Callianassa affinis* (10–20 mm Hg) by Thompson and Pritchard (1969).

Under asphyxial conditions both *Typhlogobius* and *Gillichthys* rapidly metabolize gasbladder oxygen, reducing the oxygen concentration to 1 vol% or less within 2–3 hours. Gasbladders of each of the species studied had filled volumes of about 4 ml per 100 g body weight. Oxygen concentrations averaged 25–35 vol%, so the quantity of oxygen contained in the gasbladder of a typical 1 g fish would be about 10 μl (4% body volume; 25 vol% O_2). Calculated on the basis of the oxygen uptake rates established by respirometry, reasborbed oxygen would have been sufficient to meet approximately one-half of the oxygen requirements of *Typhlogobius* and one-fifth or less of the requirements of *Gillichthys* during the first hour of asphysia. Considering the limited probable quantity of the other oxygen stores in the blood and tissue fluids, it is unlikely that the blood could retain any appreciable amount of oxygen beyond the third or fourth hour of asphyxia.

Many fishes are known to resorb oxygen from the gasbladder when asphyxiated (Jones, 1957). However, the respiratory significance of this oxygen has been questioned. Black (1942) expressed the opinion that respiratory benefit to the tissues was unlikely, because in euphysoclists blood from the gasbladder returns directly to the heart and is mixed with deoxygenated venous blood. Steen (1963) pointed out that this mixed blood must pass through the gills before reaching the tissues, and that oxygen would diffuse from partially oxygenated blood to oxygen-free water. A diffusive loss of oxygen could be prevented by not perfusing the gills or by shunting the blood away from the gill lamellae (Fange, 1966). This may be why *Typhlogobius* and *Gillichthys* immediately slow or stop gill ventilation when exposed to anoxic water, although energy conservation would be an important additional benefit. Non-respiratory shunts have been found in the gill filaments (Steen and Kruyse, 1964), but their function in asphyxia has not been investigated.

Although the blood of *Gillichthys* and *Typhlogobius* is almost certainly anoxic after the third or fourth of asphyxia, both species tolerate asphyxia for much longer periods. *Gillichthys* survives for 6.5 to 12.5 hours in anoxic seawater, while *Typhlogobius* survives for up to 60–94 hours. During this time maintenance energy must be derived from anaerobic metabolism.

Accumulation of lactate by anoxic specimens of *Typhlogobius* indicates that energy is produced by anaerobic glycolysis. Each μM of glucose-1-phosphate (from glycogen) which is fermented to 2 μM of lactic acid produces 3 μM of adenosine triphosphate (ATP). Under aerobic conditions, oxidation of one μM of glucose-1-phosphate produces 39 μM of ATP and utilizes 5 μM of oxygen. Therefore, anaerobic production of 1μM of lactate is energetically equivalent to the consumption of 0.23 μM, or 5.2 μl, of oxygen in aerobic respiration. The 95% confidence limits for the slope of the lactate accumulation regression are 0.90 to 1.41 μM/g/hr. The resting oxygen uptake rate of a 1.5 g *Typhlogobius* is 14 μl/g/hr, so 95% confidence limits for anaerobic energy production are equivalent to 33–52% of the aerobic rate.

If lactate production by *Typhlogobius* corresponds quantitatively to glycogen utilization, then the overall anaerobic energy metabolism is reduced to half or less of the aerobic metabolism. It is known that energy metabolism is reduced in diving birds and mammals, probably as a result of mass-action blocking of metabolic pathways in uncirculated tissues (Scholander, 1964). The possibility also exists that end-products of anaerobic metabolism other than lactate may be formed. Various acid end-products, such as acetic, proprionic, and succinic acids are produced by the anaerobic metabolism of molluscs (Mehlman and von Brand, 1951; Hammen, 1969), while roundworms (Saz and Weil, 1960) channel glycogen into fatty acids. Although these or other processes may play a minor role in the anaerobic metabolism of *Typhlogobius,* it is probable that production of lactic acid is the dominant energy-yielding process.

Oxygen is generally considered essential for maintenance of life in vertebrates. While some tissues tolerate anoxia relatively well, other tissues are rapidly damaged or killed. The brain is most vulnerable in this respect. The vulnerability of the vertebrate brain to anoxia is related to the high energy requirements of nerve cells, and energy requirements are correlated with the maintenance of steep ionic gradients across nerve cell membranes (Robin, Vester, Murdaugh and Millen, 1964). Maintenance energy is ordinarily derived by oxidative phosphorylation, as evidenced by the high oxygen consumption of brain tissue, and by the rapid loss of function when oxygen is denied. In some cases it is possible for nerve cells to preserve excitability in the absence of oxygen, if the enzymes of anaerobic glycolysis are present in adequate concentrations, and if an adequate supply of glucose is available (Lampert, 1961). Verzhbinskaya (1952) has pointed out that the brain tissue of fishes contains higher concentrations of glycolytic enzymes than the brain tissue of other vertebrate groups.

The brain of a few vertebrates is apparently capable of extended anaerobiosis. Belkin (1963; 1968) found that some species of turtle would continue to breathe nitrogen for 12 to 24 hours or longer. Foetal and newborn mammals of some species also have a remarkable anaerobic capability (Dawes, 1968). Anoxic survival time of both turtles and newborn mammals is drastically shortened by iodoacetate poisoning, demonstrating the importance of anaerobic glycolysis (Belkin, 1962; Himwich, Berstein, Herrlich, Chester and Fazekas, 1942). A continued exchange of metabolites, presumably glucose and lactic acid, between circulating blood and brain is presumed to be vital for extended anaerobic survival (Belkin, 1968; Dawes, 1968).

The central nervous system of *Typhlogobius* may be especially well-suited to function anaerobically because of its relative simplicity. The brain is proportionally quite small, even in comparison with the small brain of *Gillichthys.* The reduced size of the brain is probably correlated with the overall poor development of sensory modalities (Ritter, 1893; MacGinitie. 1939). Histological examination indicates a poor development of neuronal layers, nuclei and fiber tracts (Scheich, Honnegger, Warrell, and Kennedy, 1973). Electrical activity is low; EEG activity can not be recorded, and evoked potentials are barely detectable by special amplifying techniques (H. Scheich, personal communication). Expenditure of energy for maintenance of ionic gradients must be correspondingly reduced.

A recent study compared the cross-sectional area of open capillaries in the brain of rapidly frozen anxoic and normoxic *Typhlogobius* (Scheich *et al.,* 1972). An extremely rich capillary bed was observed in stained brain sections. The area of open capillaries in anoxic fish averaged 2.4 times greater than in normoxic controls, due primarily to an increase in mean capillary diameter. The resulting five fold increase in capillary surface area would greatly facilitate the exchange of metabolites between blood and brain tissue, and may represent an important adaptation for anaerobic brain metabolism.

Extended anaerobiosis requires not only energy production for maintenance of vital functions, but also some means of counteracting the rise in hydrogen ion concentration resulting from accumulation of lactic and pyruvic acids or other acid metabolites (Beadle, 1961). Turtles, which have large buffer stores, apparently buffer anaerobically generated hydrogen ion (Belkin, 1963; Robin, Vester, Murdaugh, and Millen, 1964). Fishes, which constantly exchange ions and metabolites with surrounding water, may be better able to eliminate excess hydrogen ion. *Typhlogobius* excretes some non-volatile acid product of anaerobic metabolism, but retains lactate ion. Since hydrogen ion and lactate ions are produced in an equimolar ratio, uncompensated loss of H^+ would soon result in an electrochemical imbalance. Evidence has been provided for NH^+_4/Na^+ and HCO^-_3/Cl^- exchange by teleost gills (Maetz and Garcia Romeu, 1964); these processes result in a net exchange of H^+ across the gills. In addition, a H^+/Na^+ exchange system (Garcia Romeu, Salibian, and Pezzani-Hernandez, 1969) has been suggested in frogs. Ionic exchange may permit *Typhlogobius* to eliminate hydrogen ion while preserving blood electroneutrality.

The available evidence suggests that survival of *Typhlogobius* in small volumes of deoxygeneated seawater is curtailed by accumulation of an excreted acid metabolite, acute toxicity becoming evident at a hydrogen ion concentration of about 1.5×10^{-6} molar (pH 5.8). In larger volumes of seawater survival is ultimately limited by some other factor, possibly eventual depletion of the carbohydrate reserves necessary for sustenance of anaerobic glycolysis.

I would like to express gratitude to Drs. Walter Garey and Robert Elsner for advice and encouragement throughout this study, and to Dr. Richard Rosenblatt for awakening my interest in the problem. Drs. Alfred Ebeling and Edward Fager read the manuscript critically and made many helpful suggestions for improvement.

SUMMARY

1. The respiratory, metabolic, and behavioral responses to asphyxia of three gobiid fishes are compared. Adult specimens of *Typhlogobius californiensis* and *Gillichthys mirabilis* are subject to hypoxic stress in their natural habitats, while *Coryphopterus nicholsii* generally is not.

2. Oxygen tensions in burrows of the ghost shrimp *Callianassa affinis,* commensal host of *Typhlogobius,* fall rapidly to nearly anoxic levels following exposure of the burrow openings by the outgoing tide.

3. The resting oxygen consumption of *Typhlogobius* is extremely low; the one gram intercept for the wet weight-oxygen uptake regression is 17 μl O_2/g/hr. The other two species consume oxygen 3 to 5 times as rapidly at rest.

4. Resting specimens of *Typhlogobius* are capable of maintaining a constant rate of oxygen uptake down to a critical ambient oxygen tension of 9–16 mm Hg. *Gillichthys* and *Coryphopterus* are capable of regulating oxygen uptake down to critical levels of 16–25 mm Hg and 19–28 mm Hg respectively.

5. Both *Typhlogobius* and *Gillichthys* metabolize gasbladder oxygen during the initial few hours of asphyxia.

6. *Typhlogobius* accumulates lactate ion during periods of prolonged asphyxia, indicating that maintenance energy is derived from anaerobic metabolism. The 95% confidence limits for the rate of lactate accumulation are energetically equivalent to 33% to 52% of the respirometrically measured aerobic metabolic rate.

7. The survival time of *Typhlogobius* in deoxygenated seawater varies with the volume of seawater. In very small volumes of seawater (< 10–20 ml), survival time is less than 24 hours. In larger volumes of seawater, survival time asymptotically approaches 80 to 100 hours. The decreased asphyxial survival time in small volumes of seawater may result from rapid accumulation of an excreted acid byproduct of anaerobic metabolism.

Gillichthys survives exposure to deoxygenated seawater for a relatively short period of time; survival times varied from 6.5 to 12.5 hours and were independent of seawater volume. *Coryphopterus* survives anoxia only 28 to 42 minutes.

8. It is hypothesized that the ability of *Typhlogobius* to function as a facultative anaerobe may be related to the low energy demand of the brain, to vascular adapta-

tions which facilitate the exchange of metabolites between brain and circulating blood, and to an ability to excrete the acid by-product(s) of anaerobic metabolism.

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THE BIOLOGY OF *ASCIDIA NIGRA* (SAVIGNY) IV. SEASONAL AND SPATIAL PATTERNS OF EMBRYONIC DEVELOPMENT AND HATCHING SUCCESS

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In previous papers one of us has drawn attention to the fact that in Jamaica *Ascidia nigra* breeds throughout the year but apparently has peaks of reproductive activity at certain times, particularly in the winter months (Goodbody, 1961, 1965). The data on which these conclusions are based were drawn from the appearance of young ascidians on test plates examined at weekly intervals. There is no accurate method of studying spawning behavior in the wild and, as the developmental time from fertilization to settlement of the larva can be as short as twelve hours, it is not possible to monitor reproductive cycles by following changes in the population of larvae in the plankton. In any case the eggs and larvae are virtually indistinguishable from certain other related species. There remains therefore an uncertainity as to whether the changing numbers of young ascidians appearing on plates is due to actual differences in the numbers of eggs released or is due to differing patterns of survival in eggs, embryos and larvae during the planktonic phase of their existence. In order to examine this possibility we have followed, on an experimental basis, the seasonal changes in embryonic survivorship when the eggs are allowed to develop in three different types of water: open ocean water, harbor water and mangrove-lagoon water.

By rearing the eggs in these different types of water we have been able to throw light on one further aspect of the biology of this species. *Ascidia nigra* is most commonly found in inshore regions both in the mangrove lagoons and in Kingston Harbour, but it is exceedingly rare to find it on coral reefs. The absence of this species from coral reefs could be due to the inability of embryos and larvae to survive in the type of water found in the open ocean and neighborhood of the reefs, or it could be due to the inability of the adult zooid to survive and grow in this environment. The experiments outlined in this paper will show that far from being unable to survive in this type of water the embryos survive better here than in water from inshore areas.

For technical reasons it was not possible in these experiments to study the survival of larvae after hatching and our data relate only to the period from fertilization to hatching. However, there is no reason to suppose that larvae will not survive in conditions in which the embryos have successfully developed, and we consider that the picture of survivorship given in this paper will apply to the whole period of early development up to the moment when the larva finally selects a site for attachment. It is also important to remember, when considering these data, that they are derived entirely from laboratory experiments under conditions free from competition or predation. The actual pattern of survivorship occuring in the wild could be quite different if there are any marked seasonal differences in predation pressure on the embryos and larvae. At present we do not know anything about the type of predation involved and nor do we know sufficient about seasonal changes in the planktonic carnivores to be able to consider this aspect of the problem.

Materials and Methods

The water used in these experiments came from three different places in the vicinity of Port Royal, Jamaica. Open Ocean water was collected from adjacent to East Middle Ground Shoal (Long. 76° 47.1′ Lat. 17° 54.6′); Port Royal water was collected from Port Royal Harbour (Long. 76° 50.6′ Lat. 17° 56.4′); Mangrove Lagoon water was collected from Fort Rocky Lagoon (Long. 76° 49.5′ Lat. 17° 56.3′). Locations can be found on a map in Goodbody (1970) or on British Admiralty Chart No. 454. Water was collected freshly each week in glass containers kept specially for this purpose. Following collection each water sample was filtered through a membrane filter (0.4 μ) and stored for use in glass containers with glass stoppers at a temperature of 28° C. The water was always used for experiments on the day following collection and filtration.

Artificial sea water was used for collecting eggs and sperm prior to fertilization. Instant Ocean (Ocean Systems Inc.) was used for this purpose. A large container was always available for use and samples for each experiment were filtered in the same way and at the same time as the other samples. As the objective of the experiments was to compare the success of development of eggs from a single animal in three different types of natural sea water it was necessary to find a "neutral" water in which to collect the eggs and sperm.

In the genus *Ascidia* eggs are shed from the ovary into a long oviduct which follows the curvature of the post-intestine and opens just posterior to the anus. When full of eggs this appears as a creamy white duct on the surface of the animal. The sperm duct accompanies the length of the oviduct, is deeper down in the tissues and is snowy white in color. After the oviduct is drained of eggs the sperm duct is clearly visible beneath. Animals used for fertilizations were first removed from the test and the heart was pierced and drained of blood, thus helping to prevent contamination of the water by blood. The animal was dried on filter paper and the oviduct pierced about half way along its length and eggs gently squeezed into a dish of water. The sperm duct was then pierced with a glass needle and the sperm drained into a separate dish of water. Fertilization was always between different animals.

Eggs and sperm collected in this way remain held in strings so that in each case it is possible to draw them back into a pipette with minimum contamination by the artificial sea water. Glass pipettes were thus used to transfer eggs from each animal into three separate dishes containing respectively Open Ocean water (OO), Harbor water (PR) and Mangrove Lagoon water (ML). Similarly the sperm was also transferred to three separate dishes. Fertilization was effected by pouring an appropriate dish of sperm (*i.e.*, from the same water type) into a dish of eggs and pouring back and forth between the dishes until the string of eggs was completely dispersed and mixed with sperm. Sperm and eggs were allowed to remain together for thirty minutes before the excess sperm was decanted off and the dish refilled with the appropriate water. All dishes were etched to indicate a level equalling 75 ml of water in the dish, so that all batches of eggs developed in a similar volume

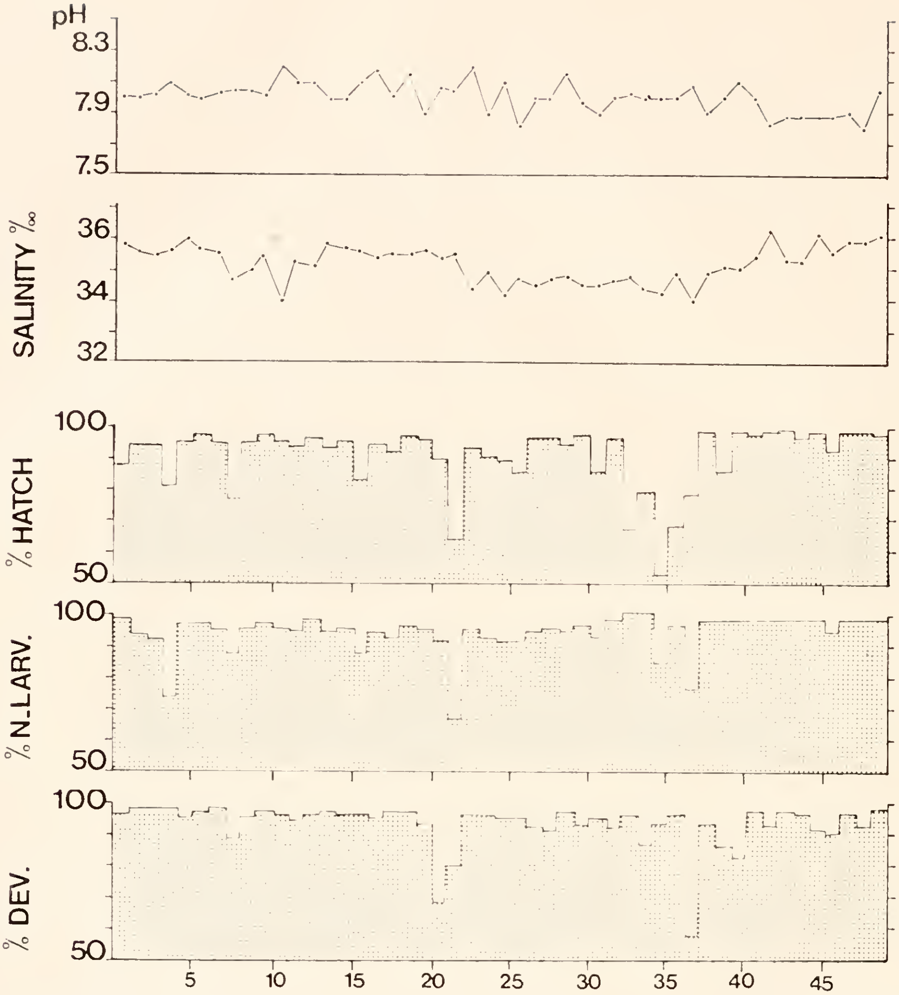

FIGURE 1. Percentage success in the development of eggs (Dev.), development of normal larvae (N-Larv.) and hatching (Hatch) in *Ascidia nigra* over a period of one year commencing April 1969 when reared in Open Ocean water. Horizontal scale shows time in weeks (week 10 = June, week 20 = September, week 30 = November, week 40 = February). Upper figures show pH and salinity of the water. All embryos were developd at 28° C (For further detail see text).

of water. Before being placed in the incubator each dish was inspected visually and the quantity of eggs reduced with a pipette until approximately 500 eggs remained, thus ensuring a similar number of eggs in each batch. Because the eggs are floating freely at this stage in a comparatively large volume of water this procedure could never be more than approximate. However, test experiments have shown that over two thousand embryos can develop successfully together in 75 ml of water without any lowering of survival.

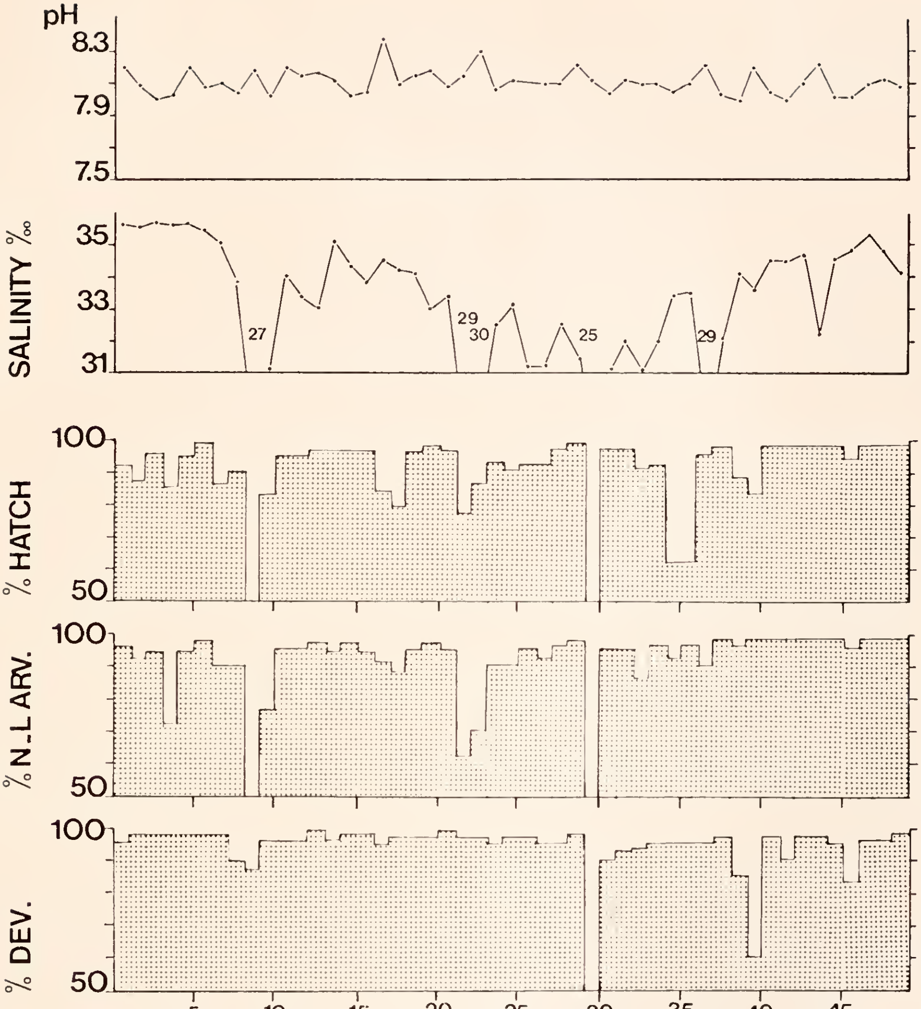

FIGURE 2. Percentage success in the development of eggs (Dev.), development of normal larvae (N-Larv.) and hatching (Hatch) in *Ascidia nigra* over a period of one year commencing April 1969 when reared in Inshore Harbor water. Horizontal scale shows time in weeks (week 10 = June, week 20 = September, week 30 = November, week 40 = February). Upper figures show pH and salinity of the water. All embryos were developed at 28° C (For further detail see text).

As soon as the number of eggs and the volume of water had been adjusted the dishes were covered with glass plates and placed in an incubator at 28° C (mean sea temperature at Port Royal). Ten hours after fertilization the contents of every dish were transferred to separate containers and fixed by the addition of neutral formalin. Each sample was later examined with an inverted microscope and total counts made of the following: (a) undeveloped eggs (b) developed eggs (c) normal

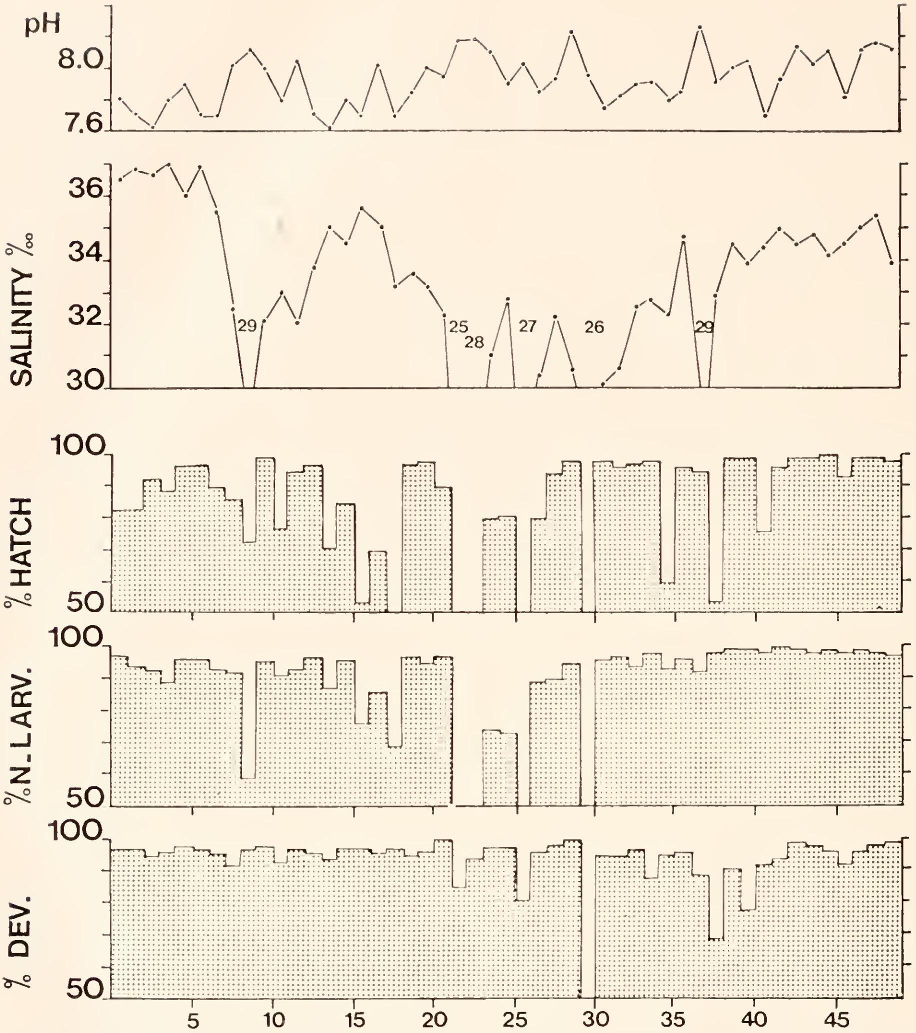

Figure 3. Percentage success in the development of eggs (Dev.), development of normal larvae (N-Larv.) and hatching (Hatch) in *Ascidia nigra* over a period of one year commencing April 1969 when reared in Mangrove Lagoon water. Horizontal scale shows time in weeks (week 10 = June, week 20 = September, week 30 = November, week 40 = February). Upper figures show pH and salinity of the water. All embryos were developed at 28° C (For further detail see text).

larvae (d) abnormal larvae (e) unhatched larvae. The data presented in this paper, and based on these figures, show only (a) the percentage of eggs which showed any development at all, (b) the number of normal larvae expressed as a percentage of the total number of eggs which developed, (c) the percentage of all larvae which successfully hatched.

All glass-ware except pipettes, used during the fertilization and development procedures was washed with Haemosol, rinsed in tap water and then in glass distilled water. Pipettes were cleaned with chromic acid and then rinsed as above.

Each weekly experiment consisted of six separate fertilizations treated as described above and using fresh animals for each new fertilization.

RESULTS

Data for forty-nine experiments carried out at approximately weekly intervals throughout a year are presented in Figures 1, 2 and 3. In each case histograms are shown to illustrate the percentage of eggs which showed any development at all: the percentage of developing eggs which produced normal larvae, whether or not they actually hatched: and the percentage of developing eggs which actually hatched. Data are also presented to show the salinity and pH of the water used on each occasion. These data may be interpreted under three separate headings.

Development

It is clear that mature eggs are available throughout the year and that some degree of development is always possible. This is most clearly illustrated in Figure 1 for eggs developing in open ocean water. If we assume that there might always be about 10% failures and consider only those occasions when less than 90% of eggs show any development, we find that serious failures of development in open ocean water only occurred in weeks 21, 22, 37 and possibly 39 and 40. In weeks 21, 22 and 37 more than 90% eggs developed in Port Royal water. In week 39, 90% eggs developed in mangrove water. In week 40 alone was there any general effect, when only 65% of all eggs developed irrespective of the water type in which they were reared. Nevertheless it is reasonable to conclude that mature eggs are always available and capable of development.

Normal larvae

Frequently ascidian larvae will develop with imperfect or distorted tails and it is these which are here considered as abnormal. It is clear that normal larvae can be produced throughout the year. In open ocean water large numbers of abnormal larvae (*i.e.* less than 90% normal larvae) occurred in weeks 4, 22, 33, 35 and 37. In weeks 4, 33, 35 and 37, 90%, or almost 90%, normal larvae were produced when developed in mangrove water. In week 22 there was a general effect and only 42% of all larvae were normal irrespective of the water type used. This is undoubtedly an environmental effect and not a physiological incapacity in the animal. The week in question was at the beginning of the rainy season and very heavy rain had occurred in the previous week, probably flushing considerable quantities of material off the land.

Hatching success

The greatest variability is to be found in hatching success, particularly in the mangrove water. Nevertheless if hatching is examined through all of the water types we find that a high degree of success is possible throughout the year. For comparative purposes we have taken 80% success as a base line and consider only major departures from this. In open ocean water such departures are to be found in weeks 8, 22, 33, 35, 36 and 37. In week 8, 85% hatched in mangrove water, in weeks 33, 36 and 37, 96% hatched in mangrove water. In week 22, almost 80% hatched in harbor water. In week 35 alone was there a fairly general departure from the base line when only 58% of all developed eggs successfully hatched irrespective of water type.

The above analysis of the data relates embryonic survival to the season of the year, but the data may also be examined so as to compare success in the three different types of water. When this is done it becomes obvious that the highest degree of success is achieved by rearing eggs in open ocean water and the least success is obtained with mangrove water (see Table I). Similarly it will be noted from Figures 1–3 that in terms of salinity and pH, the open ocean is the most stable environment while the mangrove lagoon is the least stable. In order to examine the causes of variability all instances in which hatching success is less than 75% have been examined in relation to salinity and pH of the water in question. The data are presented in Table II.

TABLE I

Overall success of development, production of normal larvae and hatching in three types of water Data are expressed as the mean percentage of all experiments carried out in the water type in question (See text).

Water type	Development	Normal larvae	Hatching
Open Ocean	92.7%	93.9%	90.3%
Harbor	92.8%	89.0%	88.0%
Mangrove	91.4%	84.4%	80.5%

The data in Figures 1–3 indicate that a high degree of survival is possible at salinities down to about 29.0 ppt but that below that survival is invariably poor. Inspection of Table II shows that in the open ocean salinities never dropped to this level, in the harbor on only two occasions out of four, and in the mangrove lagoon on four occasions out of ten. Salinity changes therefore, cannot be the only factor responsible for poor hatching success, although when salinity does fall below 29 ppt there is almost complete failure of development and hatching.

Berrill (1929) has shown that hatching in the Ascidiidae is inhibited at low pH values. In his experiments with *Phallusia mammilata* he found total inhibition below pH 6.8 but 95% of embryos hatched successfully at pH 7.2. Although values as low as this never occurred in our experiments it still remains possible that lowered pH may have been responsible for poor hatching in some instances in mangrove water (Table II). However, this cannot be the case for the instances of poor hatching in open ocean water and harbor water. Of these there was poor hatching in both water types in weeks 35 and 36 and it seems likely that there was some common factor at work. No plausible reason can be put forward for poor hatching in open ocean in week 33, but week 22 coincided with the beginning of the September rains and, as already mentioned, there may at this time have been considerable run off from the land of toxic substances such as insecticides accumulated during the dry season.

The data presented so far make it clear that *Ascidia nigra* is capable of producing viable eggs at all seasons of the year and that these eggs are able to develop successfully. However, the physiological ability of the animal to produce viable eggs does not imply that it will spawn. In an attempt to throw some light on the question of spawning cycles we kept records of the state of the gonoducts in weekly samples of twenty animals over a period of two years. An arbitary scale was used from 0 to 5 to indicate the condition of the oviduct from completely empty (0) to very distended (5). Figure 4 shows for the year 1968/69 the percentage of oviducts in each sample which were full of eggs, *i.e.,* stages 4 and 5. The animals in question were all collected in the vicinity of Port Royal Harbour and it is clear from the figure that within this population there appear to be regular cycles of activity in which the ducts fill and empty. If we assume that the sample used each week is representative of the whole population in the area, and this may be an unwarranted assumption, then it would seem that spawning is in fact continuing throughout the year. The data for the year 1969/70 showed similar but less dramatic cycles of activity.

TABLE II

Salinity and pH in relation to lowered hatching success. For explanations see text.

Water type	Week no.	Percentage hatching success	Salinity	pH
Open ocean	22	64	35.0	8.05
	33	67	34.75	8.03
	35	52	34.2	8.0
	36	68	34.9	8.0
Harbor	9	0.03	27.7	8.18
	30	0.00	25.6	8.12
	35	62	33.4	8.05
	36	63	33.5	8.10
Mangrove	9	72	29.5	8.12
	14	70	35	7.62
	16	53	35.6	7.7
	18	34.8	33.2	7.7
	22	0.00	25.7	8.17
	23	34.5	28.6	8.18
	26	3.5	27.6	8.02
	30	0.0	26.3	8.00
	35	58	32.3	7.8
	38	53	32.9	7.68

DISCUSSION

The data presented in this paper can leave no doubt about the fact that mature eggs of *Ascidia nigra* are available throughout the year in Jamaica and that there

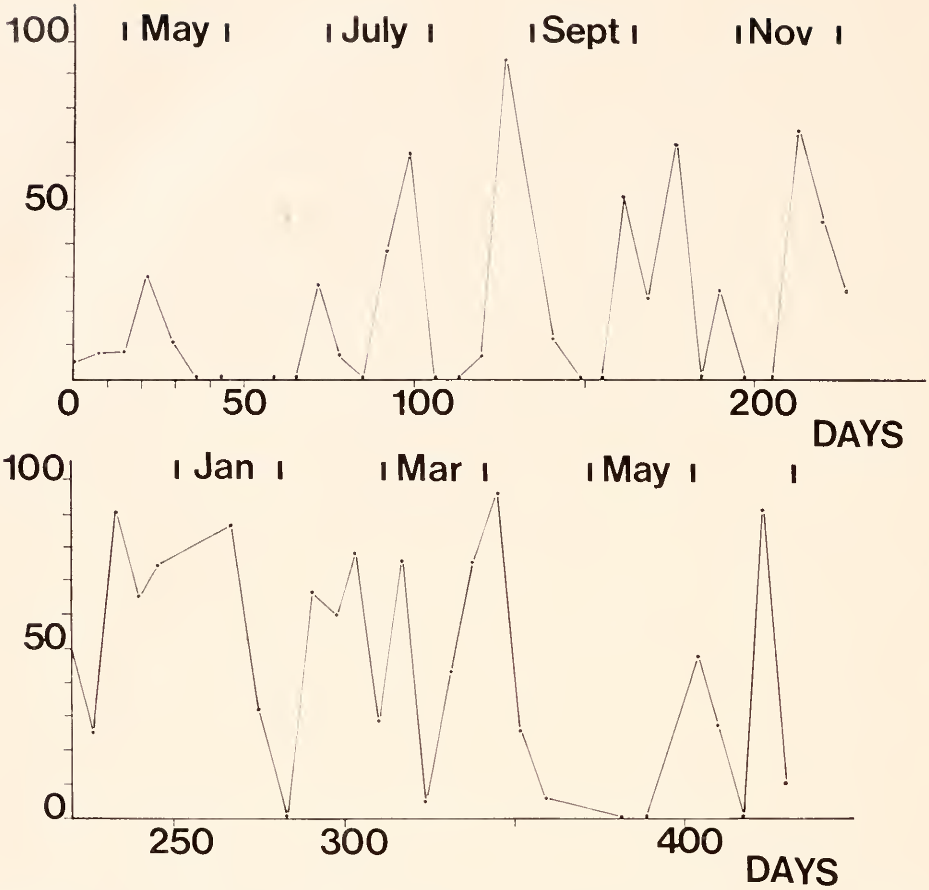

Figure 4. Percentage of gonoducts in stages 4 and 5 (*i.e.* full and distended with genital products) in weekly samples of *Ascidia nigra* from April 1968 to June 1969.

is no season of the year when, for physiological reasons, such eggs are unable to develop. This is most clearly demonstrated when eggs are developed in open ocean water, in which there are very few occasions when survival was poor. When survival was poor in open ocean water survival was usually high in one of the other water types.

Nevertheless the fact that viable eggs are always available and can always develop successfully does not in itself permit us to conclude that the fluctuations in numbers of young ascidians appearing on test panels, as illustrated by Goodbody (1961, 1965), is not due to seasonal differences in reproduction but must be due to other causes. It is still possible that, while mature eggs are always available, animals do not actually spawn at all seasons. The cyclical nature of activity in the gonoducts, which is also confirmed from older unpublished data, is difficult to interpret. It might indicate that spawning is wholly cyclical: that eggs are produced continuously to fill the duct and that this is followed by total emptying of the duct in a single process. Alternatively, it may be that when the duct is full there is a continuous discharge of gametes at a relatively slow rate, keeping pace with egg production. In the latter case the presence of empty ducts would indicate the aftermath of a sudden burst of increased activity. A great deal more work needs to be done to elucidate the precise nature and timing of spawning cycles in this species.

On the basis of the data now available to us we may conclude that in this species in Jamaica spawning occurs throughout the year on a cyclical basis and that this may in part account for the seasonal differences in settlement of larvae already referred to. There are no seasonal differences in patterns of survivorship in embryos which might in any way account for the observed patterns of larval settlement. In another paper (Goodbody and Gibson, 1974) it will be shown that differences in the patterns of survival in post-metamorphic juvenile ascidians are, however, an important contributing factor in accounting for the observations recorded by Goodbody (1961, 1965).

We pointed out in the introduction to this paper that *Ascidia nigra* is very abundant in inshore harbor waters but is rare in open ocean environments such as coral reefs. The absence of the species in these environments could be due to a failure of the embryos to develop or a failure of the post-metamorphic ascidian to survive. It is clear from the data presented in this paper that there can be no inhibition of embryonic development in open ocean water and that its absence from coral reefs must be due to other factors.

The distribution of ascidians in various habitats in Jamaica including coral reefs will be discussed by one of us in a later paper, but a few general points are pertinent in relation to the survival of *Ascidia nigra*. On the reef crest and in the lagoon (*cf.* Goreau 1959 for terminology) only those species of ascidian occur which can survive underneath stones and most of these are encrusting colonial forms. On reef butresses and on the fore reef slope ascidians are rare and confined to a few species including *A. sydneiensis* and *Pyura vittata*. Many more species and individuals are to be found in the deep reef beyond 40 meters. In inshore environments *Ascidia nigra* is always found raised off the substratum on rocks, piers, boat moorings *etc.*, but never embedded under stones or within the sediments. Within the reef system one would therefore not expect to find it in shallow water but only on the fore-reef slope. Its absence from the reef may be due to competition from other organisms and in particular corals which may prey directly on the larvae before they settle. Similarly newly settled larvae will be subject to intense predation pressure by reef dwelling fish (Bakus, 1964). However there is other evidence which shows that *Ascidia nigra* may be unable to succeed in the open ocean for other reasons. Piers, boat moorings and marker buoys within the harbor system are usually heavily encrusted with *A. nigra* but such structures placed outside of the harbor seldom have this species attached to them. *A. nigra* is probably very dependent on phytoplankton for its food and requires large quantities such as are only to be found in very productive waters, as in the harbor and lagoons.

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SUMMARY

1. The development of embryos of *Ascidia nigra* has been studied over a period of one year. Embryos were reared in water taken from three distinct areas, the open ocean, inshore harbor and mangrove lagoon.

2. Eggs are available throughout the year and are always capable of successful development. Spawning probably occurs on a cyclical basis at least once per month.

3. Embryos develop and hatch more successfully in open ocean water than in inshore waters. Differences in success of development and hatching are in part accounted for by differences in salinity and pH, but other factors may also be involved.

4. There are no distinctive seasonal differences in embryonic survival.

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THE BIOLOGY OF *ASCIDIA NIGRA* (SAVIGNY) V. SURVIVAL IN POPULATIONS SETTLED AT DIFFERENT TIMES OF THE YEAR

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In previous papers one of us has reported on certain aspects of the biology of populations of the tropical ascidian *Ascidia nigra* with particular reference to reproduction, growth and survival in Jamaica (Goodbody, 1961a, 1962, 1963a, 1963b, 1965). Settlement of larval *Ascidia nigra* has been shown to go on throughout the year indicating that reproduction must be continuous within the whole population. Settlement data are based on the appearance of young ascidians either within existing populations or on test plates and they show marked variability with a tendency for larger numbers of new animals to appear in the winter than in summer. This variability could be due to increased egg production or better survival in the winter months of embryos, larvae or later stages of the ascidian. Studies on egg production are difficult to achieve and at present it is only possible to examine aspects of survivorship. Goodbody and Fisher (1974) have reported on survival of embryos and the present paper is concerned with the question of seasonal survival of juvenile and adult populations of ascidians.

During the first few weeks of life after metamorphosis of the larva there is a great variation in survivorship and Goodbody (1963a) illustrated three basic types of survival curve representing populations which left no survivors, about 3% survivors and about 15% survivors after six weeks of life. Goodbody assumed that these differences between populations were due to biotic effects of the surrounding community and suggested that interference by barnacles and algae was the most likely factor contributing to mortality. Once a population is established the mortality rate is low until about the fifteenth month after settlement following which it rapidly increases so that most animals are dead after about twenty months (Goodbody, 1962). These earlier studies were based on only a few experiments and provided no information on any seasonal differences in patterns of survivorship. If such differences were to exist they might have an important bearing on cycles of reproductive activity and the number of larvae available for settlement at any given time.

The work outlined in the present paper sets out to examine the following questions. (1) Is there any seasonal pattern of survivorship in juvenile populations which might account for the different densities of young animals appearing in older populations? (2) Is there any relationship between spawning peaks and survival of young ascidians which would suggest that spawning may be concentrated in periods of high survival? (3) How do abiotic and biotic factors in the environment affect survivorship? (4) Is there any evidence for seasonal differences in survival of adult populations which might suggest a selective advantage is given to individuals starting life at a particular time of the year?

The approach to answering these questions has followed two separate lines of investigation in two different sets of experiments. This is necessary because juvenile ascidians are not visible to the naked eye in the first few weeks of life and must be reared under conditions in which their substrate can be removed for examination under a microscope. Such a substrate is unsuitable for the long term study of adult populations. It has not been possible to develop suitable techniques for studying natural spawning behaviour so that the second question remains unanswered. Changing patterns of juvenile survival have been studied over a period of two years by Gibson and illustrate some small but important seasonal differences. Attempts to correlate these changes with the associated biota have met with only limited success and suggest that barnacles and filamentous algae are less important than detritus and diatoms. Survivorship and expectation of life in a number of populations of adult ascidians, which started life at different times of the year, have been studied by Goodbody over a period of three years. The differences between these populations is examined in relation to both season and the nature of the associated sessile community.

Methods

The methods used for studying juvenile populations were the same as those used in an earlier study. Larval ascidians were reared in the laboratory from artificial fertilizations and then allowed to settle on glass microscope slides. As soon as settlement was complete the slides were mounted in leucite (perspex) frames which were then suspended in the sea where natural sessile communities developed in association with the ascidians. Each frame carried six slides and was backed by black leucite so as to provide a dark background and permit growth on one side of the slide only. The frames could be removed from the sea at intervals and the slides individually examined under a microscope. For fuller details see Goodbody (1963a).

The method for obtaining and growing adult ascidian populations on "Tufnol" panels have already been outlined (Goodbody, 1962) and the specific details of the particular series of populations reported on here are given by Goodbody (1965).

Table I

Mean percentage survival of 32 pairs of populations of young specimens of Ascidia nigra *reared at four feet (1.2 meters) and at eight feet (2.4 meters) respectively below the surface of the sea. The data used in this table are drawn only from those instances in which populations commenced life simultaneously at four feet and at eight feet, thus providing matching pairs. Usually there were approximately 100 individuals in each population (see text).*

Day	5		10		20		30		40		56	
Depth (in feet)	4	8	4	8	4	8	4	8	4	8	4	8
Mean % Survival	67.3	69.8	26.7	40.3	8.3	19.1	5.8	12.5	4.4	9.9	4.3	8.9
S.D.	16.8	16.4	20	17.5	8.8	14.4	7.3	12.0	6.4	10.4	6.4	9.3
t	0.58		2.904		3.63		2.73		2.558		2.315	
P	0.6		0.005		0.001		0.010		0.02		0.02	

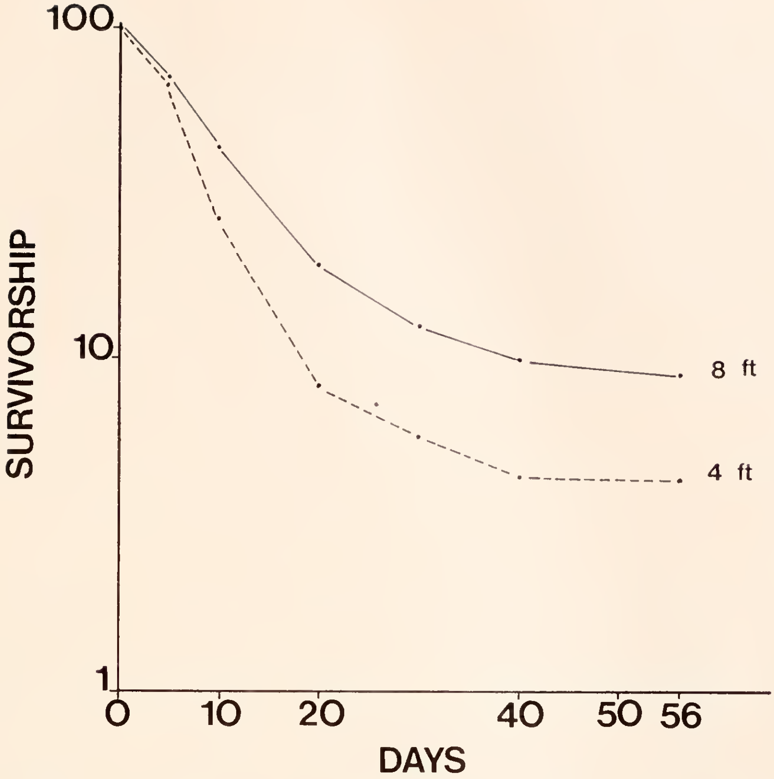


FIGURE 1. Mean survival in the first fifty-six days of life of all populations of *Ascidia nigra* reared at 4 feet (dashed line) and at 8 feet (solid line) below the sea surface. (See Table I).

Six separate populations were developed on panels immersed at two month intervals over a period of a year commencing in October 1959. Four panels were used for each population, two at four feet (1.2 meters) and two at eight feet (2.4 meters) below the sea surface. Each panel grew populations on both sides so that there are eight subpopulations within each main population. Panels were normally inspected at monthly intervals until October 1962 when the study was terminated.

Population C, which started life in February 1960, suffered serious damage and handling loss in the winter of 1960/61 and data from it are not used in this study on survival. Populations A and B (October 1959 and December 1959) suffered storm damage in the winter of 1960/61 and the data have been adjusted to enable survival curves to be drawn and an estimate made of the expectation of life. For details of the method of adjustment see Goodbody (1962). No observations were made in November 1960 during a period of exceptionally high winds. Figures given in the tables for this month are based on the mean values for October and December respectively. For details of the presentation of life tables and the calculation of the expectation of further life see Deevey (1947).

RESULTS

Survival in juveniles

Between January 1964 and December 1965 a total of 74 juvenile populations were studied, 35 reared at 4 feet (1.2 meters) and 39 at 8 feet (2.4 meters) below the surface of the sea. Each population was checked at intervals of about three days for a total duration of 56 days (8 weeks) after settlement of the larvae. Populations were selected which had as near to 100 individuals as possible. The mean population size at 4 feet was 89.6 (S.D. ± 13.1) and at 8 feet was 90.1 (S.D. ± 16.0). The plotted survival curves for each of these populations are deposited in the library of the University of the West Indies (Gibson, 1967) and no attempt is made to reproduce them here. All three types of survivorship recorded by Goodbody (1963a) occurred amongst these populations and as a general rule the pattern of survival is determined by the twentieth day after settlement (Gibson, 1967). The data in these survival curves have been used to construct both Table I and Figure 3.

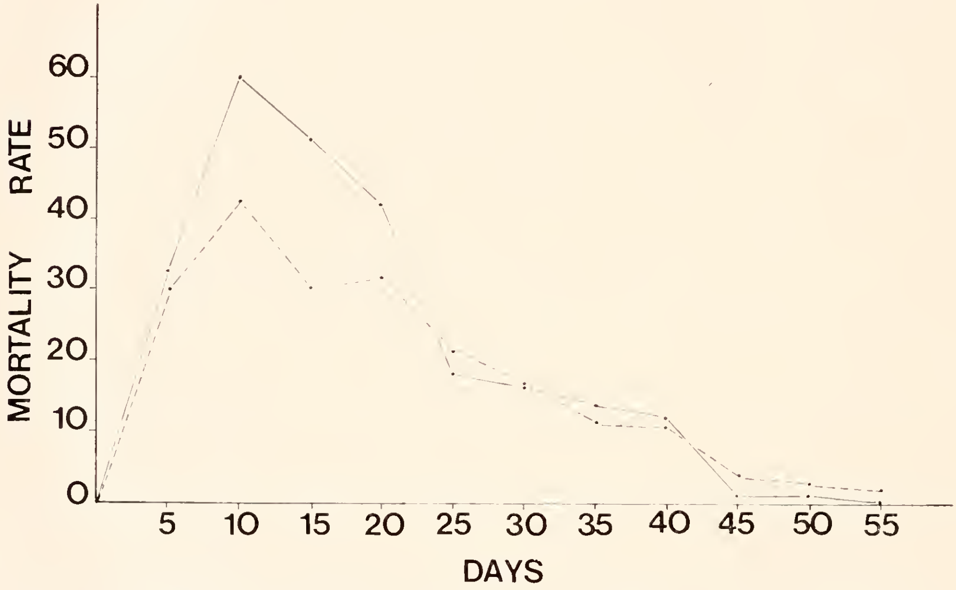


FIGURE 2. Mortality rate (100 q_x) of *Ascidia nigra* in the first eight weeks of life.

Table I shows for 32 populations at 4 feet and 32 populations at 8 feet the mean percentage survival on the 5th, 10th, 20th, 30th, 40th, 50th and 56th day after settlement. These data can be used to construct the overall survival curves for 4 feet and 8 feet illustrated in Figure 1. This shows that in general more than 75% of ascidians have died before the nineteenth day when the black pigment is normally established (Goodbody, 1963a) and they confirm the contention that the first three weeks of life are the most critical in the life of the animal after settlement. This is illustrated more forcefully in Figure 2 where the data have been re-calculated so as to show the mortality rate (*i.e.*, the number of animals dying in each interval of time per hundred alive at the beginning of that interval). A second and more important point, which emerges from Table I, is that there is a marked difference in mean survival between populations reared at 4 feet and at 8 feet and that this difference is significant from the 10th day onward. There may thus be some survival value in settling at the deeper level and the meaning of this is discussed later on.

In Figure 3 the percentage of each population surviving to the 56th day is plotted on a seasonal basis, the data above the base line being populations reared at 4 feet, those below the base line being reared at 8 feet. The dotted lines above and below the base line represent the two means of survival at the respective depths. Normally two populations were reared at each depth in each month and roughly two weeks apart. On certain occasions it was not possible to rear one or both groups and in order to differentiate these from periods of zero survival on the plot they are shown as lightly shaded bars extending to the mean.

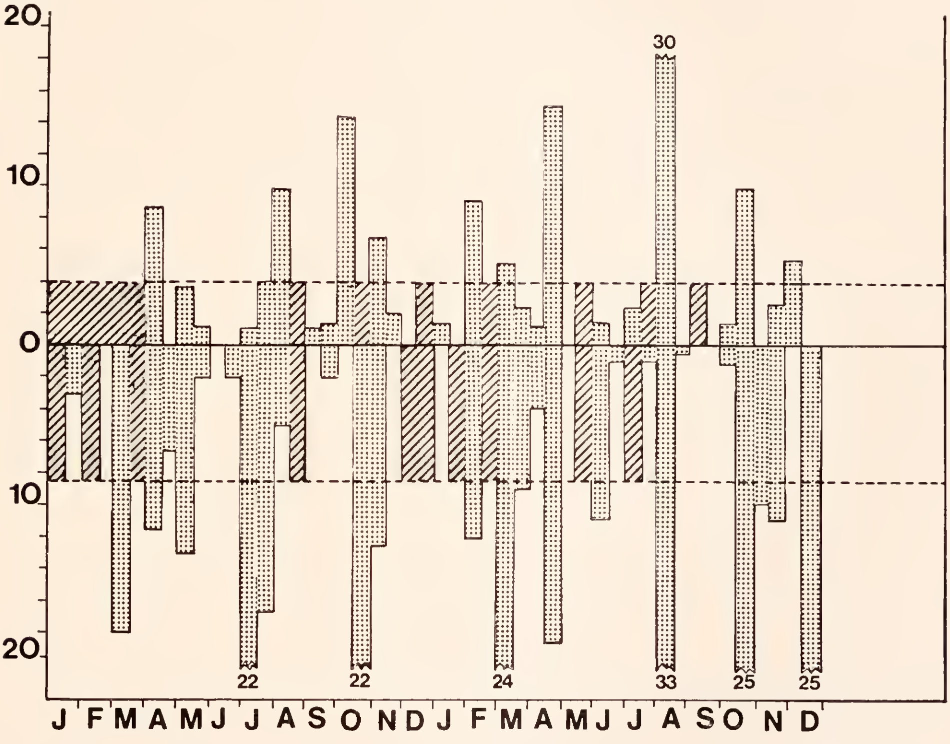
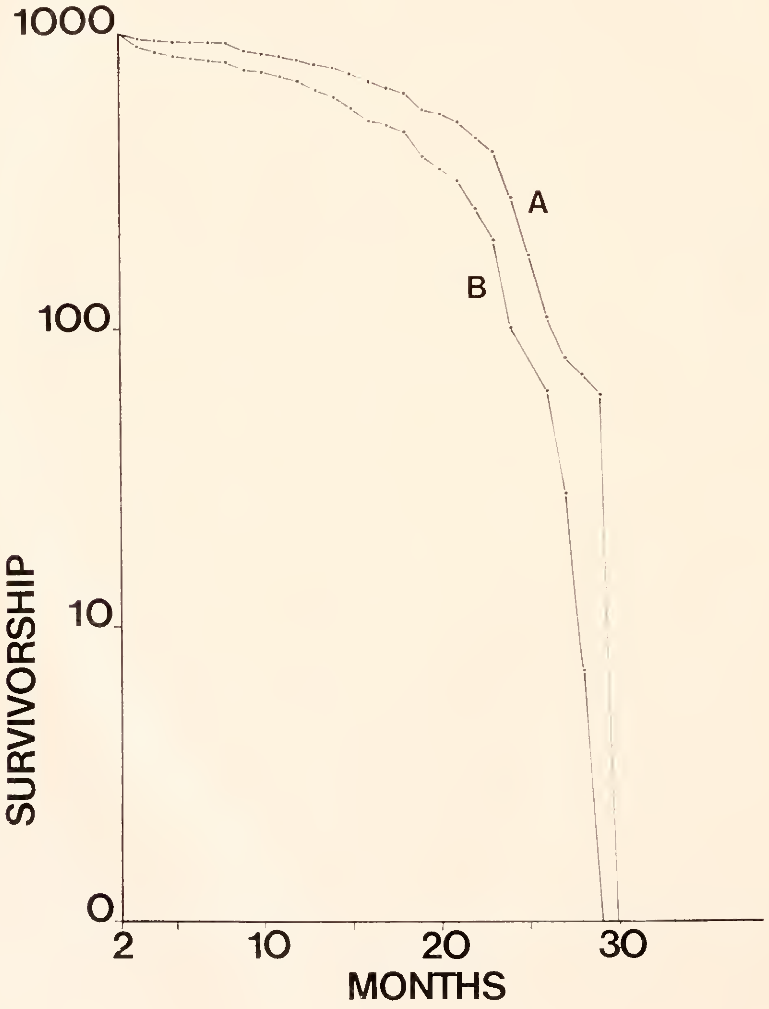


FIGURE 3. The percentage survival of ascidian populations on the fifty-sixth day after settlement when commencing life at different times of the year, shown as dotted blocks. Data above the base line refer to populations reared at 4 feet (1.2 meters) below the surface; data below the base line refer to populations reared at 8 feet (2.4 meters) below the surface. The dashed line indicates mean survival at each level. Cross-hatched blocks are periods in which no observations were made. Unfilled blocks indicate zero survival. For further explanation see text.

It is apparent from this figure that there are two periods of poor survival in the year centered around the months of June and September respectively. There may also be a period of poor survival in December but the data for 1964 are too few to make this certain. Outside of these periods there is considerable variation in survivorship and it is apparent that high survival at 4 feet is usually matched by high survival at 8 feet, thus suggesting a general rather than a localized environmental effect on the populations.

Survivorship and expectation of life in adult populations

In an earlier paper (Goodbody, 1962) survivorship was plotted separately for each cohort of animals appearing in the first three months and these were treated as separate populations. The differences were small and in this paper only sur-



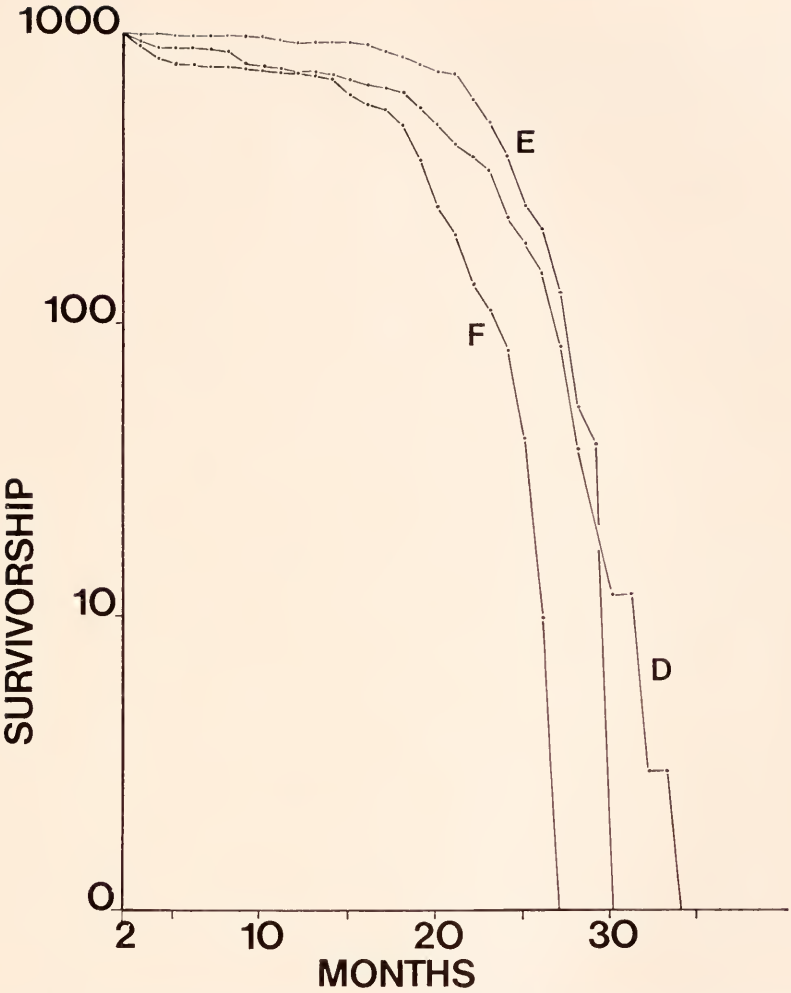


Figure 4. Survival curves for populations of *Ascidia nigra* settling at different times of the year. Graphs commence when animals are approximately two months old: (a) populations settled in October (A) and December (B); (b) populations settled in April (D), June (E) and August (F).

vival for the total number of adult *Ascidia nigra* present in each population two months after first immersion of the panels has been plotted.

Table II shows for each adult population the month of the year in which panels were first immersed, the total number of animals included in each population and the survival of these populations as cohorts of 1000 animals. It also shows the expectation of further life in each population in every month.

Survival curves for these five populations are shown in Figure 4. They confirm the picture given earlier (Goodbody, 1962) and show that irrespective of the time of year at which populations commence life the pattern of survival is the same.

TABLE II

Survivorship (l_x) and expectation of further life (e_x) for five populations of Ascidia nigra *settled at different times of year. Survivorship is expressed for cohorts of 1000 animals and the actual number of animals in each population is given in parentheses at the head of each column. Expectation of life is given in months.*

Population	A Oct (145)		B Dec (463)		D April (341)		E June (77)		F Aug (196)	
Time in Months	l_x	e_x	l_x	e_x	l_x	e_x	l_x	e_x	l_x	e_x
2	1000	16.9	1000	13.4	1000	15.9	1000	20.1	1000	13.1
3	952	16.7	899	13.8	935	15.9	1000	19.1	910	13.4
4	945	15.8	862	13.4	891	15.7	1000	18.1	821	13.8
5	938	14.9	836	12.8	886	14.8	993	17.3	786	13.3
6	924	14.2	815	12.1	886	13.8	987	16.4	776	12.5
7	924	13.2	806	11.3	875	12.9	987	15.4	765	11.7
8	924	12.2	791	10.5	865	12.1	987	14.4	765	10.7
9	869	11.9	750	10.0	789	12.2	987	13.4	755	9.8
10	848	11.2	737	9.2	771	11.5	974	12.5	745	8.9
11	834	10.4	710	8.5	751	10.8	948	11.9	730	8.1
12	807	9.2	683	7.8	733	10.1	935	11.0	730	7.1
13	786	8.9	640	7.3	730	9.1	935	10.0	719	6.2
14	766	8.2	606	6.7	716	8.3	935	9.0	699	5.4
15	726	7.6	558	6.2	698	7.5	935	8.0	612	5.1
16	687	7.0	501	5.9	663	6.8	922	7.1	571	4.4
17	647	6.4	483	5.1	651	5.9	870	6.5	546	3.6
18	627	5.6	461	4.3	625	5.2	831	5.8	480	3.0
19	548	5.3	380	4.1	557	4.7	779	5.2	367	2.8
20	529	4.5	348	3.5	487	4.4	740	4.4	255	2.8
21	499	3.7	314	2.8	416	4.1	727	3.5	204	2.4
22	438	3.2	253	2.3	381	3.4	584	3.2	138	2.3
23	399	2.4	197	1.9	340	2.7	494	2.7	112	1.7
24	279	2.3	98	2.3	232	2.7	377	2.4	82	1.1
25	179	2.3	76	1.8	191	2.2	260	2.2	41	0.7
26	110	2.4	62	1.1	150	1.7	221	1.5	10	0.5
27	80	2.1	28	0.7	85	1.5	130	1.2	0	—
28	70	1.4	7	0.5	38	1.8	52	1.2		
29	60	0.5	0	—	21	1.9	39	0.5		
30	0	—			12	2.0	0	—		
31					12	1.0				
32					3	1.5				
33					3	0.5				
34					0	—				

Survival is high in the first fifteen months of life after which there is a rapid decline and most animals are dead after about two years. This decline in the population and its relevance to physiological and ecological factors is discussed later on.

Comparisons of populations of this sort are more meaningful if expressed in terms of the expectation of further life (e_x) at any given moment of time. In continuously reproducing species such a comparison can give a measure of the relative contribution each population may make to the total number of larvae produced and

TABLE III

Ascidia nigra; *expectation of further life* (e_x) *in various populations in relation to density and time of settlement. A to F are the principal populations used in the present study, and L.S. (D), (E) and (F) are late settlers in the main populations. M is a population studied in an earlier experiment* (cf. *Goodbody, 1962*)

Population	Settlement date	No. animals	Density/sq m	e_x
A	Oct. 59	145	129	16.9
B	Dec. 59	464	414	13.4
C	Feb. 60	228	204	—
D	April 60	341	304	15.9
E	June 60	77	69	20.1
F	Aug. 60	196	175	13.1
M	Aug. 57	327	146	12.0
L.S. (D)	Nov. 60	34		9.1
L.S. (E)	Nov. 60	114		10.25
L.S. (F)	Nov. 60	34		11.1

hence to survival of the species. The expectation of life of these populations, of the original study population in 1957 and of three late settled populations in the present study are presented in Table III. If for the time being we exclude the data on late settled populations (L.S.) and examine only the data for the five main populations of 1959/60 and the one for 1957, it is apparent that there is considerable

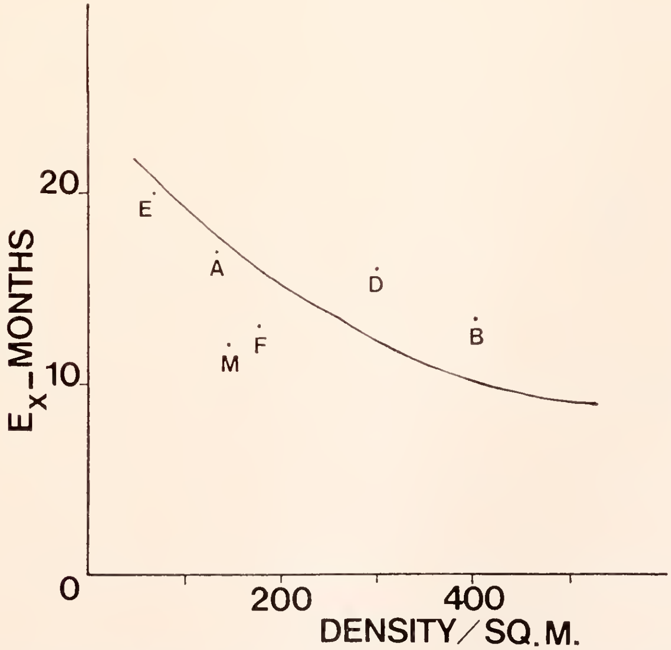


FIGURE 5. Relation between expectation of life (e_x) and density in six populations of *Ascidia nigra* (see text). The line is a trend line and is not calculated.

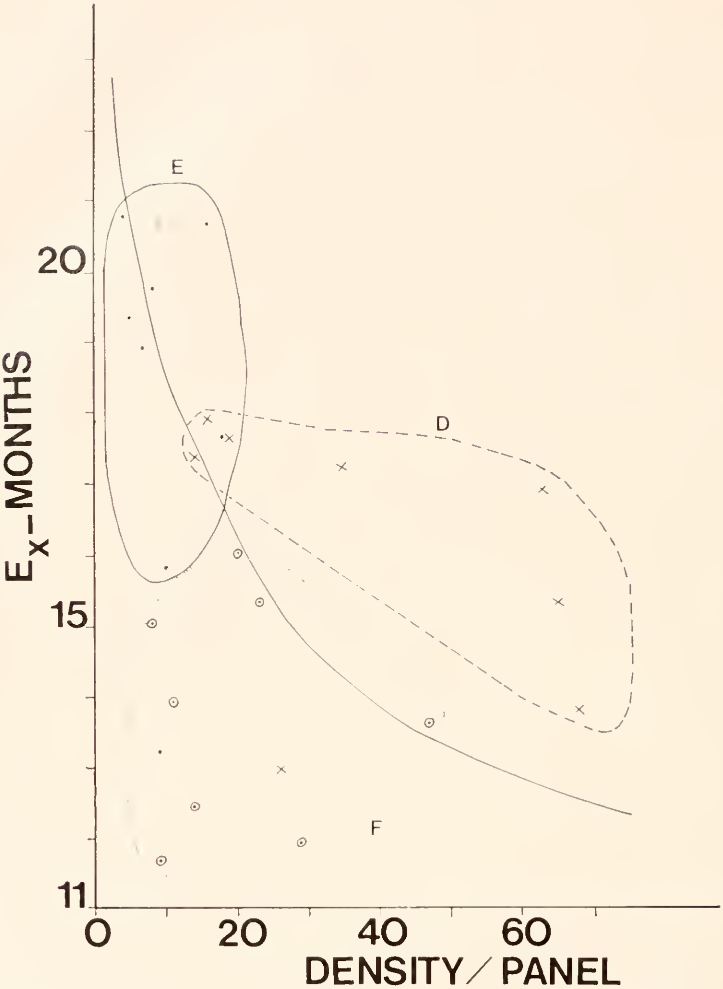


FIGURE 6. Relation between expectation of life (e_x) and density in various sub-populations of *Ascidia nigra.* The curved line is a trend line only and is not calculated. Sub-populations belonging to population D (April settlement) are enclosed by a dashed line; populations E (June) is enclosed by a solid line and population F (August) is enclosed by a dotted line (for further explanation see text).

variation in expectation of life between different populations. The data are insufficient on which to draw conclusions concerning seasonal variation, but it is apparent from simple inspection that there may be a relationship between density and expectation of life; this is plotted in Figure 5 and suggests a curvilinear relationship. However, this must be treated with caution as there are many other factors, ecological and physiological, governing mortality.

Each population is in reality composed of a series of sub-populations represented by the eight separate panel surfaces on which the communities are growing.

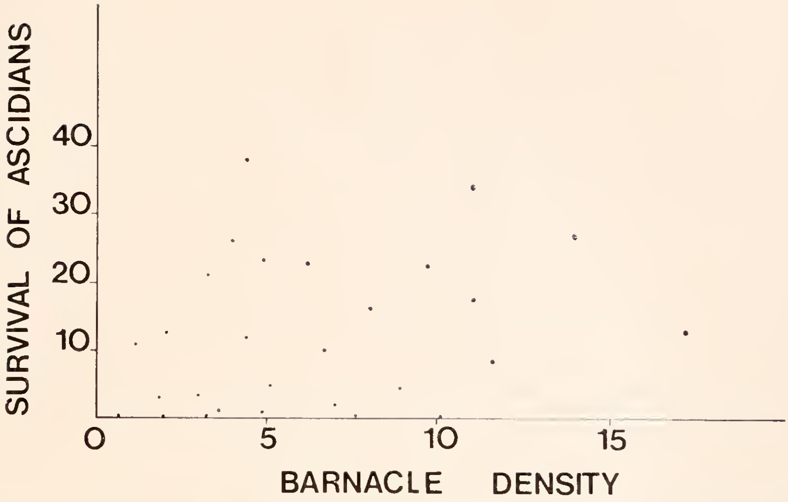


FIGURE 7. Relation between maximum recorded density of barnacles per square centimeter and the survival of ascidians on the twenty-first day after settlement. Survival is expressed as a percentage of the original population (see text).

This enables us to look at density effects in a different way by calculating the expectation of life for each of the sub-populations and plotting these against density. Data for 24 such groupings are plotted graphically in Figure 6. Only data from populations D, E and F are used in this analysis, and no data are drawn from the damaged populations of A and B. There is again a suggestion of a curvilinear relationship but the points are too widely spread for any definite curve to be drawn. This indicates, of course, that factors other than density are operating to control survival and is made clear by drawing lines to enclose the points from each of the main populations, D, E and F which are now seen to occupy separate positions on the plot.

We can then only tentatively conclude that survival and expectation of life are influenced by density but that other forces are modifying or controlling this effect. These are discussed below.

Cause of mortality

Goodbody (1963a) suggested a number of factors which might contribute to mortality in juvenile ascidians but did not identify any one thing which might cause such marked differences between populations as are illustrated by the data presented in this paper. He suggested that competition from barnacles and interference by growths of filametous algae might be important factors as well as overgrowth by colonial ascidians.

In the present study records were kept in the first year of the changes in populations of barnacles and the growth of filamentous algae but there is no evidence at all that either of these was contributing in an important way to changes in survivorship of ascidans. Since maximum settlement and activity of barnacles occurs between the 7th, and 14th days after immersion of a panel we have examined the

TABLE IV

Suspended matter in the sea at Port Royal, 1965. Measurements are in milligrams per liter (Gibson, 1967)

Month	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
No. of Observations	5	8	9	5	9	8	7	8	6
Mean	4.6	4.9	4.3	5.3	5.4	5.75	4.3	4.9	6.85
S.D.	1.7	0.9	1.6	0.6	0.85	1.53	2.5	1.48	4.78

relationship between ascidian survival at the 21st day and the maximum density of barnacles occurring in the community (Figure 7). It is apparent that high densities of barnacles cannot be held responsible for high mortality in certain ascidian populations. On the contrary there is an indication that there might be a direct relationship between high survival of ascidians and high densities (*i.e.,* high survival) of barnacles, although if this is true the relationship is obscured by other modifying factors.

Similarly if survivorship is plotted against growth of *Enteromorpha flexuosa*, the dominant filamentous algae, there is no apparent correlation at all (see Gibson, 1967, for further details). It seems therefore that while the presence of barnacles and filamentous algae may contribute to ascidian mortality by modifying the environment, neither is of sufficient importance to account for the wide fluctuation in survivorship so frequently observed. However, while we were studying these populations it became apparent that there was another variable which might be of

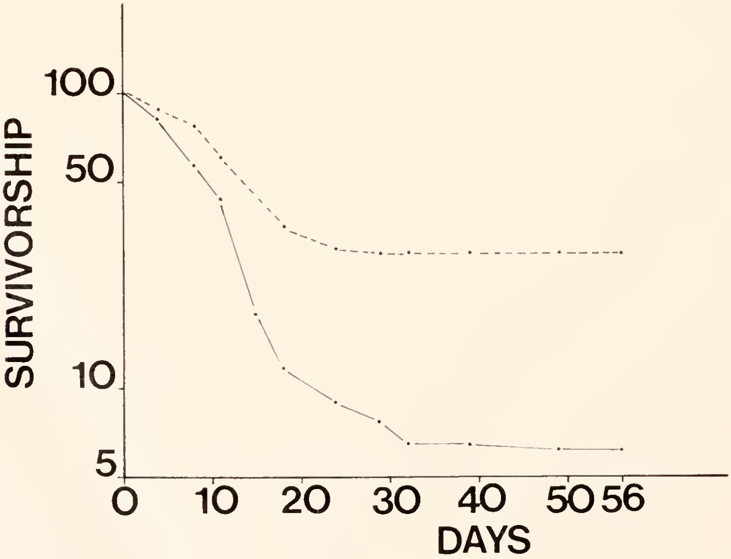


FIGURE 8. Survival of hooded (broken line) and unhooded (solid line) populations of *Ascidia nigra* during the first eight weeks of life, when reared at four feet (1.2 meters) below the sea surface.

more importance than any of those previously considered, and this was the accumulation of particulate matter and benthic diatoms in such a way as to produce a blanketing effect on the whole community.

On the basis of 65 observations, made between April and December 1965, Gibson (1967) showed that the quantity of suspended material in the sea off Port Royal varied from 0.5 to 16.3 mg/liter. In the absence of data on the composition of this material and the nutritional requirements of *Ascidia nigra* it is impossible to say how this might affect survival. The mean monthly values shown by Gibson and reproduced in Table IV do not indicate any trend that might account for changes in survival. Nevertheless observation has shown that at times large quantities of material accumulate on communities and that considerable growths of diatoms notably *Licmophora, Diatoma* and *Baccilaria* are associated with these deposits, the whole appearing as a "blanket" on the community.

The significance of these accumulations became apparent too late to enable quantitative measurements to be made in the present study. However Gibson (1967) carried out some experiments in which populations of young ascidians were grown in the normal way on glass slides but in which the whole frame holding the slides was protected by a black leucite (perspex) hood. The effect of this is of course to cut down the available light and reduce algal growth. Two effects were noted in these experiments. The development of the blanket of diatoms and other material was strongly inhibited in comparison to replicate populations growing unshielded, and similarly survival was better in the populations shielded by a hood than in those which were unshielded.

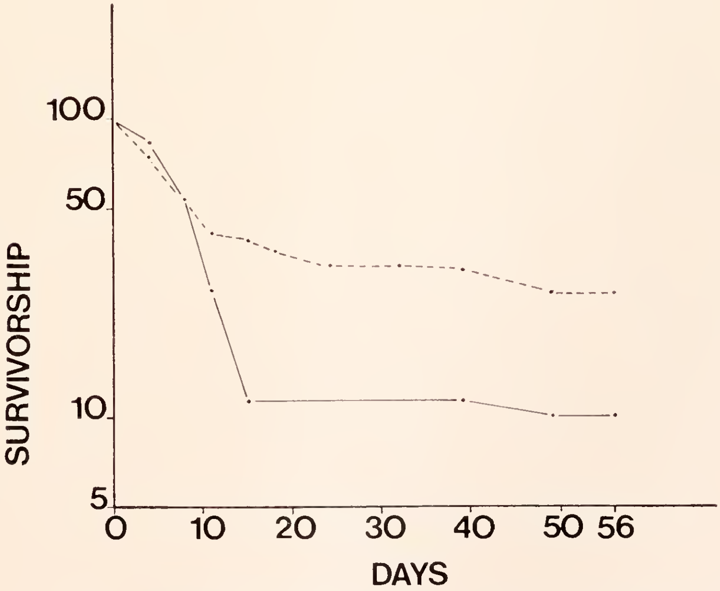


FIGURE 9. Survival of hooded (broken line) and unhooded (solid line) populations of *Ascidia nigra* during the first eight weeks of life when reared at eight feet (2.4 meters) below the sea surface.

Only two such replicate populations have been studied, one at 8 feet and the other at 4 feet below the surface. Survival curves for these pairs are shown in Figures 8 and 9 and show clearly the type of effect which was observed. The data are too few from which to draw meaningful conclusions and we have not been able to follow up this interesting line of approach. However, the data do suggest that large accumulations of benthic diatoms may be an important factor in controlling ascidian populations at this stage of their life and research toward substantiating this and elucidating the factors which control these diatom populations is very desirable.

Ascidians which survive beyond the first few weeks of juvenile life have a high expectation of further life and in the absence of predators it is not easy at first to see what may cause the decline of populations after the first 15 to 20 months of life. In an earlier paper (Goodbody, 1962) the possibility of senescence and physiological death in ascidians was discussed and attention drawn to the fact that in all cases studied ascidians appeared to have a limited life span and that this might be governed by physiological factors. However the decline in the population of *Ascidia nigra* was shown to coincide with the development of the climax sessile community, notably the development of sponges. There are thus three major possible components of mortality in ascidian populations: (1) Physiological death or senescence; (2) Intra-specific competition and (3) Inter-specific competition.

Senescence implies that given ideal environmental conditions and freedom from competition an animal will ultimately die of old age and that there is a maximum age beyond which an individual is unlikely to survive. Senescence in animals has been discussed by Comfort (1956). It is difficult to prove in wild populations where environmental stresses are modifying the life span in various ways. It could be studied in ascidians in aquaria provided an adequate food supply was available. It has not been possible to maintain *Ascidia nigra* in a healthy condition in the aquarium as it appears to be dependent on phytoplankton for its food supply. However the related species *A. interrupta* grows fairly freely in the aquarium and two populations were maintained in large wooden troughs for the whole of their life span, free from competition from other organisms. A steady stream of fresh sea water pumped directly from the sea was maintained through the troughs and appeared to provide adequate food. These populations, derived from artificial fertilizations were set up for another purpose and apart from initial counts no record is available of deaths in the first ten months of life of one population and five months of the other. Nevertheless it is possible to draw survival curves for these two populations and to demonstrate that each has a negative skew similar to those in Figure 4, one lasting for 950 days, the other for 770 days. However since both populations died at about the same time, in spite of commencing life five months apart, there is a suspicion that other factors besides senescence may have been involved. For the time being therefore we cannot be certain that senescence occurs in tropical ascidians and further effort must be directed towards this end.

Intra-specific competition among adult ascidians is also difficult to assess. The data presented earlier suggest that survivorship may be affected by density and if this is correct, presumably it must be due to direct competition for food in densely crowded populations. However crowding may in certain circumstances be beneficial by preventing the growth of other competing species. This point is discussed further below.

The most important factor limiting survival of *Ascidia nigra* appears to be the growth of the surrounding sessile community and particularly the competing effects of sponges and bivalves. In the very early stages of community development *A. nigra* is associated with colonial species of ascidian and sometimes solitary stolidobranch ascidians both Styelids and Pyurids, but there is no evidence at all that these compete for food. They do compete for space and in early development fast growing colonial didemnids may overgrow and smother the solitary forms (Goodbody, 1963a).

The period of ascidian dominance in the community lasts for approximately twelve months after which bivalves and sponges become increasingly important as filter feeders and therefore increasingly competitive with the ascidians. The first twelve months of stability is followed by a decline in ascidians as the sponges and bivalves reach their climax. It is not possible to assess the relative importance of sponges and bivalves as competitors, but it is easy to visualize why sponges are so successful. In a filtering ascidian the area of collection of food is restricted to a small zone around the branchial siphon while in a sponge food collection is a direct function of the surface area. Provided an ascidian can maintain its siphonal collecting zone beyond the limit of sponge growth it may survive, but once the sponge grows beyond this it is likely to "trap" and filter all water in the neighborhood (Goodbody, 1962).

During the course of these investigations detailed notes and photographs were kept of the monthly changes taking place in the communities. In order to assess the impact of the climax sponge community on life expectation in ascidians the populations D, E and F have been examined to see what difference, if any, occurred between populations. On the basis of their expectation of life we should expect that the climax may have been reached more quickly in F ($e_x = 13.1$) than in D ($e_x = 15.9$) which should be more rapid than E ($e_x = 20.1$). The detailed analysis is too bulky to reproduce here but may be summarized as follows. In D sponges had penetrated noticeably by month 6 (M6), were common by M10 and dominant by M15 to M16. In E sponge had penetrated by M6, was not considered significant until M11 but did not fully take control until M19; they never became so common as in D and F. In F sponge had penetrated by M4, was common by M8 and had a dominant foothold by M11/12.

It would seem that survival in E may have been enhanced by the relatively slow development of sponge but suppressed in F by the early and extensive growth of sponge. In D sponge developed at about the same time as in F but the much greater density of ascidians made expansion more diffcult and perhaps served a protective function. We thus have an anomalous situation in which a high density of ascidians may be a disadvantage in terms of intra-specific competition but may also be an advantage in staving off the competition of sponges and even bivalves. In the case of population D the growth of solitary ascidians consisted of dense aggregations of both *A. nigra* and Stolidobranch ascidians of several species which between them left little space for sponge colonization and development.

DISCUSSION

Data presented in this paper confirm that survivorship in the first two months of life is exceedingly variable and may range from zero to 33% of the original population of settled larvae. This variability is not markedly seasonal but it is possible to identify two periods of exceptionally poor survival, one centered around June and the other around September. The mid-summer period is of particular interest as it has previously been demonstrated that natural settlement is minimal at this period (Goodbody, 1961a, 1965) and unpublished data on gonadial cycles suggest that reproductive activity as a whole is depressed between April and July. However, this lead cannot be pursued any further until far more is known about spawning cycles. We have not been able to ascertain for certain what are the causes of mortality in this "critical period" after metamorphosis but predation, smothering by fast growing colonial ascidians and interference by filamentous algae and excessive blankets of diatoms and fine particulate material appear all to be important factors. Blankets of diatoms have also been found to be an important cause of mortality in the temperate water ascidian *Corella willmeriana* (Lambert, 1968), and they may explain the differences between survival of young ascidians at 8 feet and at 4 feet below the surface. Similar environmental factors must be operating at both levels as periods of good survival at 8 feet are normally matched by periods of good survival at 4 feet, but in general more than twice as many animals survive to two months of age at 8 feet as at 4 feet. The water at Port Royal is heavily laden with suspended material and light penetration is poor. The mean of 14 secchi disc readings taken over a period of one year was 4.2 meters (± 1.44 meters). Such a value suggests that the difference in illumination may be sufficient to cause significant differences in diatom growth at the two levels of our panels, 1.2 meters and 2.4 meters.

It is obvious that food supply might also be limiting on certain occasions but at present we have no data at all on the particular nutritional requirements of young ascidians. Nevertheless, it is unlikely that food *per se* is ever limiting, as the water is so heavily laden with suspended material, but the availability of this food might be affected by interference from other organisms preventing a free flow to the ascidian. Alternatively, turbulence might cause the ascidian to close its siphons repeatedly and halt the filtration process. These are effects which at present we cannot measure.

Once the black pigment is developed mortality decreases sharply and from the fourth week onward populations remain fairly stable for about a year (Goodbody, 1962 and present paper). This stability can be attributed to a lack of predation and relative freedom from competition. Apparently the test substance is distasteful (Goodbody, 1962) and hence the freedom from predation. In dense aggregations of *Ascidia nigra* intra-specfic competition may be important and result in a density dependent relationship. Competition between different species of ascidian has not been studied. We know (Goodbody, 1967) that within the genus *Ascidia* there is sufficient ecological isolation to prevent competition, but the effect of dense aggregations of stolidobranch ascidians such as *Pyura vittata* and *Microcosmus exasperatus* on less specilized forms such as *Ascidia nigra* is unknown. There is nothing in the present study to suggest competition between the two except for space, but studies on the feeding ecology of these forms would help to clarify the issue.

The major influence on populations of adult *Ascidia nigra* still seems to be the developing communities of sponges and bivalves which reach dominance, if not their climax, about fifteen months after a new community has started to develop. Although sponge communities are known to act as inhibitors to the development of sessile communities (Goodbody, 1961b) their effect on adult ascidian populations is most likely direct competition for food as suggested earlier in this paper. Furthermore this competitive exclusion is apparently confined to the less specialized phlebobranchiate ascidians such as *A. nigra* and stolidobranchs continue to thrive in amongst the sponge communities. When the communities discussed in this paper were finally removed after three years there were stolidobranch ascidians still living there. This is a reflection of the difference in branchial structure and hence ability to filter the water (Croxall, 1971).

The effect of sponges is demonstrated more clearly if we examine all of the data in Table II where the populations of late settling *Ascidia nigra* are compared in terms of their expectation of life with the original populations amongst which they have settled. These late populations are derived from a total of 182 new animals which settled in the populations D, E and F in October and November 1960 as the result of unusually high survival of young ascidians in established communities. All of the late settled populations have a considerably shorter expectation of life than the originals but the two populations (early and late settled) have tended to die at about the same time. We interpret this as being due to the effect of the surrounding community.

There remains the question of senescence. Clearly under natural conditions few specimens of *Ascidia nigra* have an opportunity to grow old but are excluded by competition much earlier. The longest survival in these experiments was an animal which lived for 33 months as against a mean expectation of life for all populations of 15.9 months. In the laboratory study of the related species *A. interrupta* the longest survival was 900 days (about 30 months) and in spite of the uncertainty in that study, it seems that three years is likely to be the maximum longevity obtainable in these animals.

Comparable data to show the expectation of life in other species of ascidian are not available, but there is ample evidence to show that amongst solitary forms a life span of between one and two years is the general rule, irrespective of latitude (Table V). *Corella willmeriana* is an exception which appears to live for no more than eight months (Lambert, 1968). It is probably also true that solitary ascidians are in general primary colonizers and poor competitors in the face of more efficient filter feeders such as bivalves and sponges. However, the potential for exploiting the environment in this way is considerably greater in tropical than in temperate water species. Tropical species are able to reproduce throughout the year and hence larvae are always available and able to exploit new situations, but in higher latitudes breeding seasons are restricted (Millar, 1971) so that larvae are only available for a few months of the year, when many other organisms are also competing for space.

Deevey (1947) predicted that marine species of animals having pelagic eggs and larvae should exhibit positively skew rectangular survival curves in which there is extremely high mortality beginning in early life, but the few individuals which survive to advanced ages have a relatively high expectation of further life. Our data confirm that this is likely true for *Ascidia nigra,* although we do not have data for the pelagic phase itself. Data given in this paper show that on average

TABLE V

Duration of life in various species of ascidian

Species	Locality	Life-span	Reference
Ciona intestinalis	Scotland	1–1½ years	Millar, 1952
	Sweden	1 year	Dybern, 1965
	Venice	1 year	Sabbadin, 1957
	Mediterranean	2 years	Pérès, 1946
Ascidia mentula	Norway	3 years	Dybern, 1969a
Ascidia nigra	Jamaica	2–3 years	Present work
Ascidia interrupta	Jamaica	2–3 years	Present work
Ascidiella aspersa	Scotland	1–1½ years	Millar, 1952
	Norway	2–3 years	Dybern, 1969a
Ascidiella scabra	Baltic	12–14 months	Dybern, 1969b
Chelyosoma macleayanum	Alaska	4 years	Huntsman, 1921
Chelyosoma productum	British Columbia	3 years	Huntsman, 1921
Chelyosoma columbianum	British Columbia	1 year	Huntsman, 1921
Corella parallelogramma	Norway	1 year	Dybern, 1969a
Corella willmeriana	Washington	3–8 months	Lambert, 1968
Dendrodoa grossularia	Britain	1½–2 years	Millar, 1954
Styela coriacea	Baltic	1½–2 years	Diehl, 1957
Styela plicata	Venice	1 year	Sabbadin, 1957
Molgula manhattensis	Venice	1 year	Sabbadin, 1957

only 6.6% of metamorphosing larvae survive to eight weeks of age. It is likely that survival in the pelagic phase, which lasts for only a day, is even less. Figure 10 has been drawn on the assumption that 0.5% of fertile eggs ultimately reach the stage of settlement and metamorphosis, but even if that estimate is grossly incorrect it will make little difference to the fact that the overall survival is as predicted by Deevey (1947).

There are still relatively few other data on survivorship in marine invertebrates. Hatton (1938) and Connell (1961) have both illustrated life table data for barnacles; Sutherland (1970) has studied the dynamics of populations of the limpet *Acmaea scabra* and Frank (1965) *Acmaea digitalis*. These are all intertidal organisms and the environmental stresses on populations are therefore totally different to those in *Ascidia*. Walne (1961) showed that oysters (*Ostrea edulis*) living at the low water mark of spring tides in Wales exhibit a positively skew survival curve but the life span is about twelve years and the period of heavy mortality lasts throughout the first eighteen months of life after which the mortality rate is very low. This type of survival curve is very similar to that of *Ascidia* although the time scale is completely different. Paine (1963) has analyzed the dynamics of populations of the brachiopod *Glottidia pyramidata* in Western Florida. *Glottidia* is a benthic filter feeder living in sand bars consolidated by marine grasses. Unlike *Ascidia* the pelagic larval life lasts for about three weeks and Paine has made quantitative estimates to show that there is in fact a massive mortality in this phase of the life history. However, the subsequent survival curve approximates to Deevey's (1947) diagonal type thus suggesting that there is a constant loss from the population rather than a stable population which dies quite suddenly as appears to be the case in *Ascidia*.

Figure 10 illustrates the differences between these two animals by showing Paine's original survival curve for *Glottidia* and a composite survival curve for *Ascidia nigra.* The latter curve is based on population E in the present study for adult animals and uses a mean survival of 6.6% for the juvenile phase in the first two months; it also assumes a survival of only 0.5% in the pelagic phase. This latter figure has no basis in fact and is used merely to complete the graph. The actual value for survival in the pelagic phase is unimportant for comparative purposes and a difference of one order of magnitude would not alter the picture illustrated. The important point is that once the pelagic phase is complete the patterns of survival in the two animals are quite different and probably reflect differences in the type of mortality affecting the two species. *Glottidia* is actively preyed upon

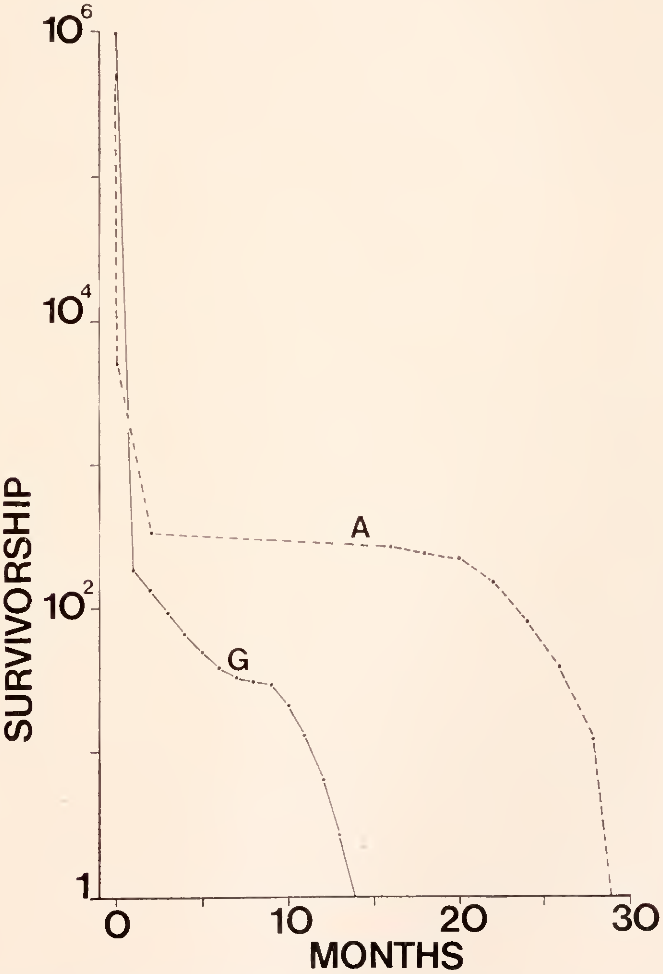


FIGURE 10. Survivorship curves for *Ascidia nigra* (Jamaica) (A), and the brachiopod *Glottidia pyramidata* (Florida) (G). Data for the pelagic phase of *Ascidia* are assumed, the remainder of the data taken from the present work. Data for *Glottidia* are from Paine (1963).

by birds and perhaps by other organisms and there appears to be an almost constant percentage loss from the population regardless of size. *Ascidia,* like *Ostrea,* appears to have no important predators in adult life and the population remains stable until near the end of its life when, as suggested earlier, the competitive action of other species brings about the onset of death. It is possible that *Ascidia* and *Ostrea* may be unusual in this respect and that the majority of marine invertebrates have life tables similar to *Glottidia,* but data are needed on many more species before positive conclusions can be drawn.

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SUMMARY

1. The survival of populations of juvenile ascidians in the first two months of life, and adults of two months and older has been studied on a seasonal basis in Jamaica, West Indies.

2. The greatest mortality takes place during the first three weeks of life during which period animals growing at eight feet (2.4 meters) have a significantly better survival than those at four feet (1.2 meters).

3. Seasonal differences in the survival of juvenile populations are not pronounced but periods of poor survival are centered around June and September. Outside of these months survivorship is very variable but similar patterns of change occur at depths of four feet and eight feet.

4. Survival and expectation of life of adults are not affected by the season of the year but are affected by density and by growth of the associated biota.

5. The blanketing effect of populations of diatoms and detritus may be a major cause of mortality in juvenile ascidians. Interference by barnacles and filamentous algae is apparently of little importance.

6. Death of adult populations is usually coincident with the time at which sponges grow level with the inhalent siphon of the ascidian and it is suggested that there is a direct competition for food between the two organisms.

7. The overall pattern of survival of *Ascidia* is discussed in relation to other animals and particularly to the branchiopod *Glottidia.* The life table of *Ascidia* may be unusual as a result of low predation in adult life.

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EFFECTS OF SALINITY ON NUTRITIONAL REQUIREMENTS OF *ARTEMIA SALINA*

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Until recently, most of the work being done on the problem of metabolism in Crustacea centered around hormonal control (McWhinnie and Corkill, 1964). The importance of nutritional state has been emphasized (McWhinnie and Kirchenberg, 1962) but very little is known about the metabolic function of nutrients. The development of an artificial medium for *Artemia* salinia by Provasoli and d'Agostino (1969) offers a good opportunity to study this problem.

Information is lacking on how salinity affects the nutritional requirements of *Artemia* (d'Agostino and Provasoli, 1968). Besides gathering some general metabolic data, a further object of this investigation was to study the morphogenetic effect of adenosine monophosphate (AMP) deficiency. AMP deficiency induces a supernumerary gonopode morphogenesis and reduces abdominal length (Hernandorena, 1970, 1972a). It is well known that *Artemia* reared at different salinities shows certain modification in form. In particular, with an increasing salinity of the medium the abdomen grows relatively longer and narrower (Gilchrist, 1960).

Extremes of salinity nonspecifically reduce incorporation of radiocarbon from 2-^{14}C sodium acetate into the intermediates of tricarboxylic acid cycle of *Artemia* (Huggins, 1969). Succinodehydrogenase activity varies with oxygen consumption in *Artemia* (Packard and Taylor, 1968) and oxygen consumption decreases with increasing salinity (Eliassen, 1952; Dutrieu, 1960; Engel and Angelovic, 1968).

So it might be expected that salinity modifies AMP metabolism and consequently morphogenesis.

Materials and Methods

The method developed by Provasoli and d'Agostino (1969) for the Utah strain axenic cultivation was used. The medium is composed of: (1) a liquid phase containing mineral salts, six amino acids, eight vitamins, two sugars and a mixture of nucleic derivatives from which guanylic acid has been omitted and has the following composition: adenylic acid, 60 mg%; uridylic acid, 10 mg%; cytidylic acid, 10 mg%; and thymidine, 5 mg%; and

(2) a fine particulate phase composed of egg albumin, rice starch and cholesterol. Duplicated experiments each using 2 × 10 ml of medium were used for all the treatments. The criteria used to evaluate the results were the growth index established according to Provasoli and d'Agostino (1969) numerical method and the survival percentage (Hernandorena, 1972a, 1972b and 1972c).

Results

The optimal starch and albumin concentrations in medium "100" by Provasoli and d'Agostino (1969) have been checked in our experimental conditions.

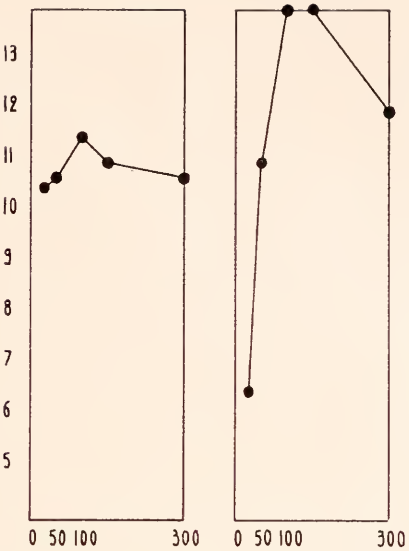

FIGURE 1. Effect of starch concentration; both abscissae represent starch in mg%; albumin constant at 20 mg%. In all the figures, growth is represented in the left-hand, and survival in the right-hand graph, the left-hand ordinate being the growth index for the 14th day of development, and the right-hand ordinate being the survival percentage for index 10 (end of larval life).

With albumin constant at 20 mg%, growth index and survival percentage increased with starch concentration up to a 100 mg% level (Fig. 1), and declined at higher concentrations. With starch constant at 100 mg%, growth index increased

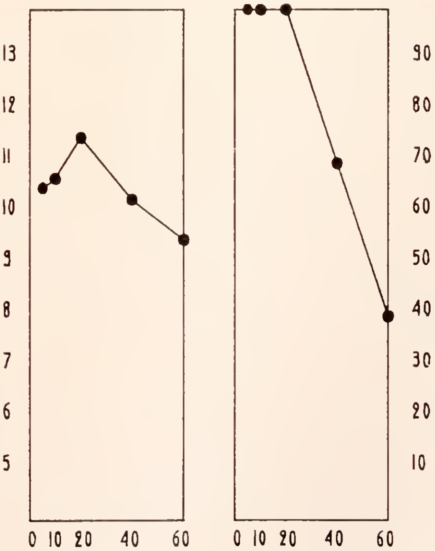

FIGURE 2. Effect of albumin concentration, both abscissae albumin in mg%; starch constant at 100 mg%; ordinates as in Figure 1.

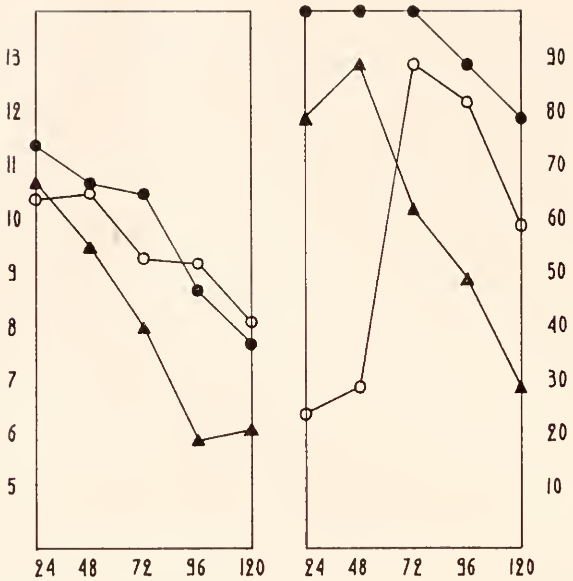

FIGURE 3. Effect of salinity on starch requirement; both abscissae NaCl in g‰; starch: open circles, 25 mg%; closed circles, 100 mg%; closed triangles, 300 mg%; ordinates as in Figure 1.

with albumin concentration up to a 20 mg% level (Fig. 2). These results are similar to those found by Provasoli and d'Agostino although albumin deficiency is partially relieved by supplementary amino acids.

Varying salinity affects the starch and albumin requirements. Starch deficiency is less detrimental and a starch concentration above optimum is more detrimental with increasing salinity (Fig. 3). Albumin deficiency is more detrimental and above optimal albumin concentration less detrimental with increasing salinity (Fig. 4).

According to Provasoli and d'Agostino (1969) as the level of albumin increases more starch is needed for optimal growth. Starch is replaceable by lecithin (Fig. 5). With starch constant at 100 mg%, lecithin is tolerated up to a 2 mg% level (Hernandorena, 1972b). Lecithin also relieves the detrimental effect of excess albumin (Fig. 6); thus confirming the need for a ratio energetic nutrient: albumin.

The optimal starch: albumin ratio stands somewhere between 5:1 and 3.3:1 (Provasoli and d'Agostino, 1969). This relationship holds true at 24‰ but not at 120‰: in a starch deficient medium (50 mg%) more albumin is needed for optimal growth (Fig. 7).

With starch constant at 100 mg%, lecithin addition (2 mg%) increases growth index at 24‰ salinity but becomes inhibitory with increasing salinity (Fig. 8) thus confirming the reduction of energetic nutrient requirement with increasing salinity.

AMP requirement depends on albumin concentration. Suboptimal AMP (20 mg%) becomes more detrimental as the albumin concentration increases (Fig. 9). In an albumin deficient medium (5 mg%) the supernumerary gonopode morpho-

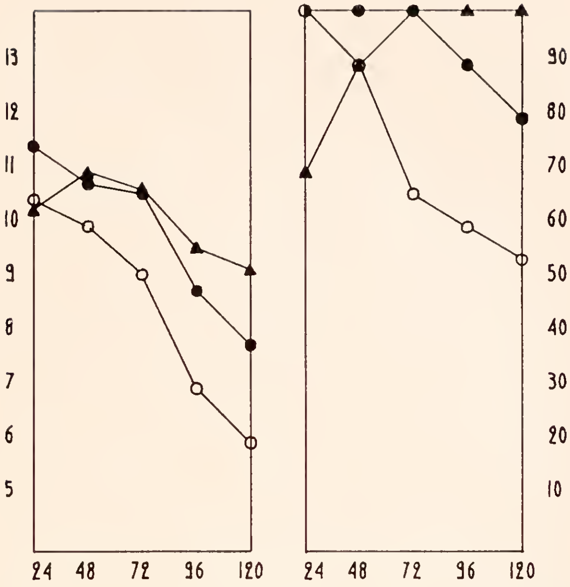

FIGURE 4. Effect of salinity on albumin requirement; both abscissae NaCl in g%; albumin: open circles, 5 mg%; closed circles, 20 mg%; closed triangles, 40 mg%; ordinates as in Figure 1.

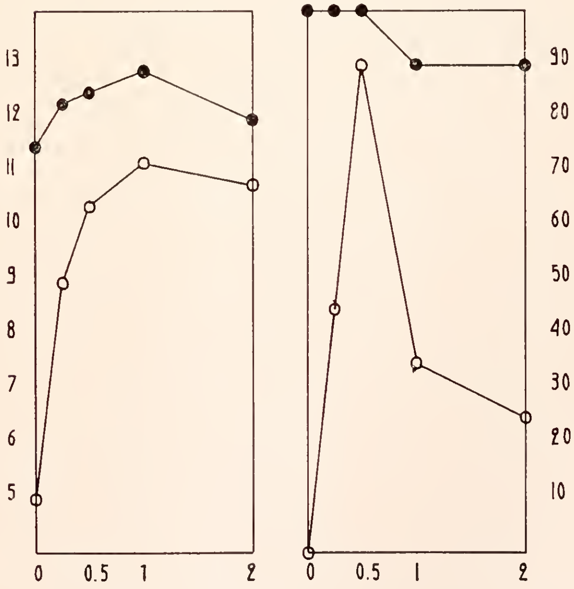

FIGURE 5. Effect of lecithin; both abscissae lecithin in mg%; open circles, no starch; closed circles, starch 100 mg%; ordinates growth and survival, see Figure 1.

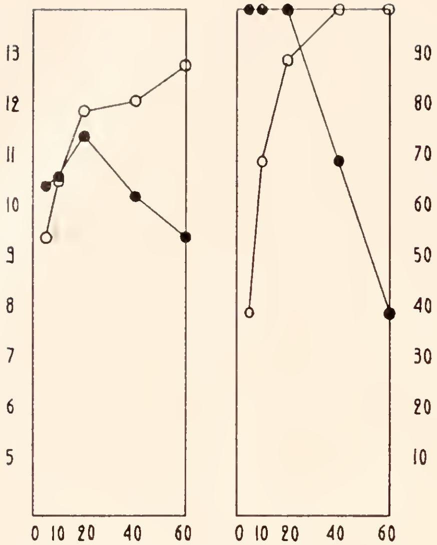

FIGURE 6. Effect of albumin concentration on lecithin requirement; both abscissae albumin in mg%; closed circles, no addition; open circles, lecithin 2 mg%; ordinates growth and survival, see Figure 1.

genesis is no longer induced by AMP deficiency. More AMP is needed for optimal growth in a medium containing an above optimal (60 mg%) albumin concentration (Fig. 10). In a starch deficient medium (up to 10 mg%) growth index and

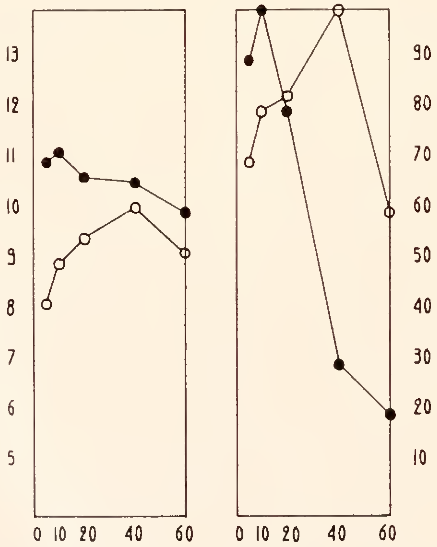

FIGURE 7. Effect of salinity on albumin requirement; both abscissae albumin in mg%; starch constant at 50 mg%; salinity: closed circles, 24%; open circles, 120%; ordinates growth and survival, see Figure 1.

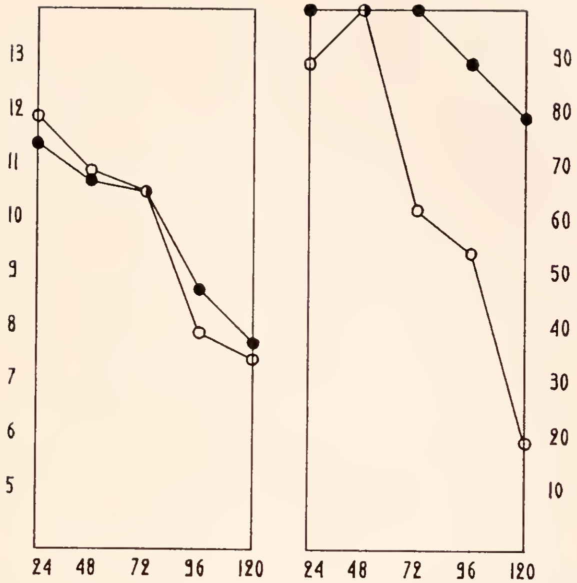

FIGURE 8. Effect of salinity on lecithin requirement; both abscissae NaCl in g‰; starch constant at 100 mg%; albumin constant at 20 mg%; AMP constant at 60 mg%; closed circles, no addition; lecithin: open circles, 2 mg%; ordinates growth and survival, see Figure 1.

survival percentage do not increase with AMP concentration. With starch constant at 100 mg%, growth index and survival percentage increase with AMP concentration up to a 140 mg% level (Fig. 11). With increasing salinity, less AMP is

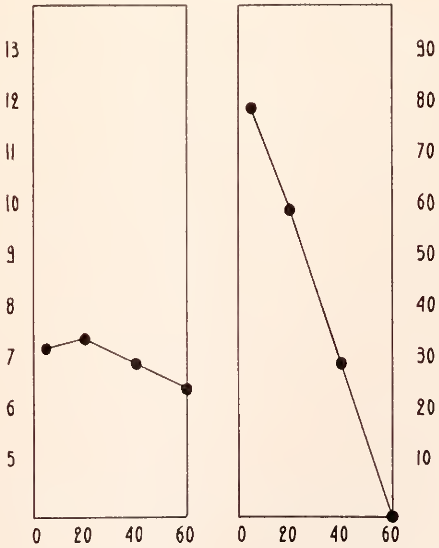

FIGURE 9. Effect of albumin concentration on AMP requirement; both abscissae albumin in mg%; AMP constant at 20 mg%; ordinates growth and survival, see Figure 1.

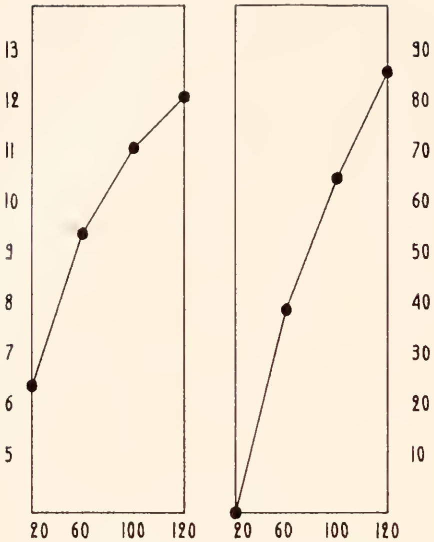

FIGURE 10. Effect of high albumin concentration on AMP requirement; both abscissae AMP in mg%; albumin constant at 60 mg%; ordinates growth and survival, see Figure 1.

needed for optimal growth. Growth index and survival percentage increase with AMP concentration up to a 100 mg% level at 72‰ and to a 60 mg% level at 120‰ salinity.

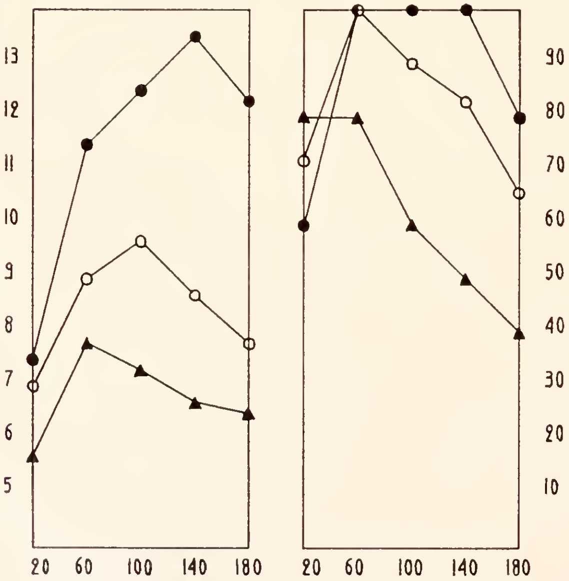

FIGURE 11. Effect of salinity on AMP requirement; both abscissae AMP in mg%; starch constant at 100 mg%; albumin constant at 20 mg%; salinity; closed circles, 24‰; open circles, 72‰; closed triangles, 120‰; ordinates growth and survival, see Figure 1.

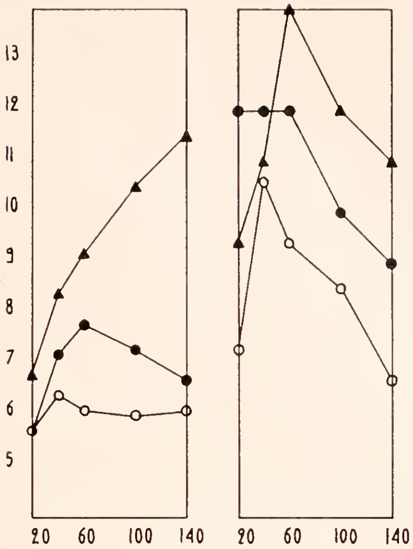

FIGURE 12. Albumin and AMP requirements at high salinity (120‰); both abscissae AMP in mg%; albumin: open circles, 5 mg%; closed circles, 20 mg%; closed triangles, 40 mg%; ordinates growth and survival, see Figure 1.

At 120‰ salinity, albumin deficiency is detrimental whatever AMP concentration. Growth index increases with AMP concentration only for above optimal (40 mg%) albumin concentration (Fig. 12). In an AMP deficient medium (20 mg%) the supernumerary gonopode morphogenesis is induced only with an albumin excess (40 mg%). These results confirm the higher albumin requirement and lower AMP requirement with increasing salinity.

DISCUSSION

The problem of energy sources in Crustacea was studied by Renaud (1949) and the results of her work led Vonk (1960) to conclude that Crustacea metabolism is mainly centered around glycogen and fatty acids. But according to Huggins and Munday (1968) the relative significance of carbohydrate in meeting the basic energy requirement still requires further investigation. The large amount of free amino acids in Crustacea and the variability of this amount under conditions leading to an energetic adjustment may be considered as indicating the use of amino acids in the process of energy production (Gilles, 1970). In *Artemia* medium the energy requirement is met by starch or lecithin at 24‰ salinity. More energetic nutrient and more AMP were needed for optimal growth as the albumin level increased. The *Artemia* purine nucleotide requirement is specific and absolute. With a given albumin concentration, growth rate and abdominal length increase with AMP concentration (Hernandorena, 1972b). In *Drosophila* developmental rate and ultimate size depend on the protein concentration of the diet (Sang, 1959). Dietary RNA requirement depends on protein concentration (Geer. 1963) and RNA requirement reflects essentially an AMP requirement (Sang, 1959). But dietary AMP requirement is not an absolute requirement in all

Drosophila strains (Hinton, Ellis and Noyes, 1951). In *Artemia* the quantitative nature of AMP requirement is relative and depends on the albumin concentration of the diet. At 24‰ salinity, the optimal growth rate is achieved with a proper energetic nutrient + AMP: albumin ratio. The protein concentration is highest during exponential growth indicating that almost all growth is due to new protein synthesis (Dagg, 1969); amino acid incorporation is used as a criterion for protein synthesis. Oxidative metabolism is essential for amino acid incorporation into tissue protein. Both glycolytic and Krebs cycle intermediates as well as ATP stimulate the incorporation of glycine into proteins of *Bombyx* (Chefurka, 1965). Since the morphogenetic effect of AMP deficiency depends on albumin concentration and salinity, the AMP morphogenetic effect should be looked for at the protein synthesis level.

With increasing salinity, starch or lecithin requirements are reduced. The incorporation of radiocarbon from 2-^{14}C sodium acetate into the intermediates of the Krebs cycle is reduced by salinity (Huggins, 1969), and as a consequence, it seems that the energy requirement is no longer met by starch or lecithin.

With increasing salinity, the albumin requirement is increased. The results of Emerson (1967) on the free amino acid level of *Artemia* nauplii incubated at different salinities led Huggins (1969) to suggest the particular importance of proline biosynthesis in adaptation to salinity by *Artemia*. Evidence has been presented supporting the use of proline as an energy reserve in *Aedes aegypti* (Thayer, 1972). Utilization of dietary amino acids in lipid synthesis by aseptically reared *Musca domestica* has been demonstrated, leucine and glutamate being the best lipid precursors (Kon and Monroe, 1971). The role of individual amino acids in adaptation to salinity and energy production by *Artemia* is under study.

Another point requires further investigation. The results of Reeve (1963) led Provasoli and d'Agostino (1969) to conclude that in medium "100" *Artemia* is ingesting particles at a maximum constant rate. *Artemia* ingested liquids but apparently to a very limited extent. Direct uptake of phosphate ion from the medium has been effectively demonstrated (Kobayashi, Saita and Tomiyama 1972). According to Croghan (1958a, 1958b) *Artemia* regulates the osmotic pressure and ionic balance of its hemolymph by taking up NaCl and water from the gut lumen and excreting NaCl through the branchiae. Osmotrophy is limited at 24‰ but may increase at 120‰ since the drinking rate of the eel has been shown to vary in relation to external salinity (Maetz and Skadhange, 1968).

SUMMARY

1. At 24‰ salinity the energy requirement of *Artemia* is met by starch or lecithin.

2. The need for a starch: albumin ratio put forward by Provasoli and d'Agostino (1969) has been extended to a need for an energetic nutrient + AMP: albumin ratio. With increasing salinity, the eneregetic nutrient requirement decreases and the albumin requirement increases.

3. AMP deficiency induces a supernumerary gonopode morphogenesis. The quantitative nature of AMP deficiency inducing the morphogenetic action depends on albumin concentration and salinity. The AMP morphogenetic effect should be looked for at the protein synthesis level.

4. The possible significance of salinity induced modifications in energetic nutrient and albumin requirements is discussed.

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LARVAL DEVELOPMENT OF THE BARNACLE *TETRACLITELLA KARANDEI* REARED IN THE LABORATORY

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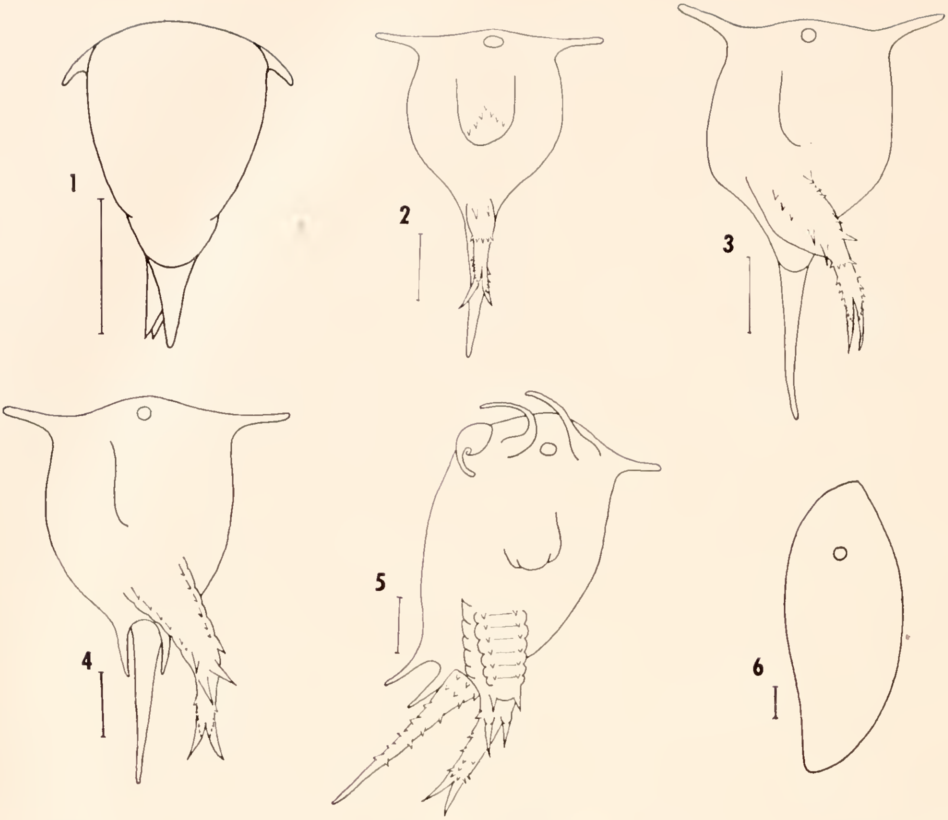
Tetraclita purpurascens Wood and *Tetraclitella karandei* Ross are two common tetraclitellan species encountered at Madh Island off the Bombay cost of India. *T. karandei* occurs on the undersurface of rocks, low in the intertidal zone, where it normally remains moist during periods of low tides. Ross (1971, page 215) observes that "ecological conditions under which this species lives and the few animals with which it is associated do not differ appreciably from those of other species of *Tertaclitella*."

The diagnostic characters of *T. karandei* as stated by Ross (1971, page 217) are "radii transversely ridged, the apical 3–4 ridges extending like fingers out and over adjoining plate, scutum transversely elongated, externally ornamented with prominent nodes where longitudinal ridges cross growth lines, intermediate articles of posterior cirri armed with three pairs of setae." Ross has cautioned that this new species is similar in many ways to the widely occurring *T. purpurascens* and records of the latter species should be re-evaluated in the light of his discovery.

The larval development of some temperate and tropical species of balanids has been studied by various workers. There has been, however, no published account of the larvae of any tetraclitid barnacles. The development of *T. karandei* was therefore examined under laboratory conditions with a view to describing each of its six nauplii and the cyprid larva. The naupliar characters such as setation of antennule, carapace sculpture, and abdominal and caudal processes have been examined and are compared with those of megabalanid, balanid, chthamalid and pedunculate species encountered in Bombay waters. Due to the paucity of the gravid specimens, it has not become possible to describe larval antennae and mandibles of *T. karandei* in the present paper. The characters described, however, are adequate to separate these larva from those of other species. Earlier Karande (1973a) reported on the larvae of *Balanus amphitrite amphitrite* and on the larvae of *Balanus variegatus* (Karande; in press, 1973b). Some of the other species examined in this laboratory are *Balanus amaryllis euamaryllis, Chthamalus withersi, Chthamalus malayensis, Ibla cumingi,* and one unidentified species of balanid. The systematic position of this unknown balanid barnacle is still being investigated.

MATERIAL AND METHODS

Small pieces of stone bearing specimens of *T. karandei* were maintained in small glass jars of natural sea water. The adults were fed daily on a diet of *Dunaliella primolecta* and the diatoms *Plurosigma* sp. and *Phaeodactylum* sp. The sea water was changed every day, and the specimens were kept dry during night hours. The embryos or the first nauplii released by these adults were reared to obtain their



FIGURES. 1–6. Larval stages of *Tetraclitella karandei;* all scale bars equal 100 μ. Figures 1, 2, 3 and 4 are first, second, third and fourth naupliar stages respectively. Figure 5 is the sixth nauplius and Figure 6 the cyprid larva.

subsequent growth stages. A general outline of the rearing technique has been earlier reported by Karande and Thomas (1971).

RESULTS

Description of the larvae

The first nauplius, usually measuring 240 μ, has a pear-shaped body (Fig. 1). Anterior horns, 50 μ long, are very clearly seen. Both the abdominal and the caudal processes are very well differentiated and each one of them measures 50 μ in length. A median eye is present. Frontal filaments have not appeared. The antennule shows a setal pattern of 0–0–0–4–2–1–1–0. The actively moving larvae transform into second nauplii in about one hour's time. Table I gives the dimensions of various cirriped larvae examined in this laboratory. Table II gives the dimensions of various appendages of *T. karandei* larvae.

The second nauplius is twice the size of the first because of a considerable elongation of the caudal process (Fig. 2). It is 480 μ long, and is almost as big as the

TABLE I

Dimensions in microns of nauplius and cyprid larvae of eight cirriped species from Bombay

Stages	*T. karandei*	*B. variegatus*	*B.a. amphitrite*	*Ch. withersi*	*Ch. malayensis*	*B. amaryllis euamaryllis*	*Balanus* sp.	*I. cumingi*
I	240	240	225	205	216	240	190	300
II	480	390	300	300	300	480	280	372
III	540	400	310	330	360	500	335	385
IV	600	425	425	360	415	600	395	420
V	700	540	460	420	420	670	480	460
VI	780	580	540	480	450	*	530	500
Cyprid, carapace	600	615	510	480	420–80	*	*	565

* Notrecorded.

second nauplius of *B.a. euamaryllis*. The latter may at times achieve a length of 500 μ and is the biggest second nauplius encountered in Bombay waters. The frontal filaments emerge but are only 30 or 40 μ long. The frontal horns are 85–90 μ long. The median lobe of the labrum is hairy and two other lobes bear very minute teeth. These teeth are missing in the subsequent stages. A highly spinulated caudal process is 200 μ long (Fig. 7, cds). The spinules are generally subequal. At two localized places, however, some spinules are slightly bigger than the rest. The base of the caudal spine is also spinulated. The abdominal process, which is shorter than the caudal process, has a well defined 80 μ long stem and two 70 μ long massive rami. At early stage, the stem has two well defined ringlets of spinules, a proximal one and a distal one. Of these the former one gets disorganized at the late second nauplius stage. The stem and the furca are thus separated by a distal ringlet of spinules. Each ramus bears a row of spines along its outer margin which runs half-way down the ramus and encircles it to form a ringlet of spinules. A distal half of the ramus is smooth. A pair of distal abdominal spines is present on a bulged abdominal process. These spines appear on a common base

TABLE II

Dimensions in microns of some larval features in Tetraclitella karandei

Larval stage	N I	N II	N III	N IV	N V	N VI	Cyprid
Total length	240	480	540	600	700	780	*
Carapace length	**	**	**	470	540	625	600
Post. carapace spine	**	**	**	80	85	110	**
Frontal filament	**	50	85	*	*	*	180
Frontal horns	50	85	85	*	*	70	**
Caudal process	50	200	270	300	300	260	**
Abdominal process	50	155	180	240	240	205	**
Distal abdominal spine	**	10	25	30	45	50	**
Proximal abdominal spine	**	**	10	10	25	35	**
Rami	25	70	85	*	*	95	**

* Not recorded.
** Absent.

and after undergoing some changes in shape and size separate into two individual, well defined spines. The setal formula for the antennule is 0–0–0–4–2–1–1–0.

The third nauplius measuring 540 μ in total length resembles the second nauplius in general morphology (Fig. 3). The frontal filaments are 85 μ long. The abdominal process measuring 180 μ, now bears proximal pair of the abdominal spines. Three pairs of segmental spines are present. The stem and the furca of the abdominal process are 95 μ and 85 μ respectively. A characteristic feature of this stage is an emergence of a preaxial seta on the antennule whose setal formula now reads 0–0–1–4–2–1–1–0.

The fourth nauplius is 600 μ long (Fig. 4). A true carapace developed at this stage is 470 μ long and 300 μ wide. The paired posterior carapace spines are 80 μ long; 120 μ long anterior horns direct ventro-laterally. The abdominal bulb, besides several minute spinules, bears a few characteristic spines each one of which is beset with minute hair(s) on its base (Fig. 8 abs). The abdomen is demarcated into segments by a series of sub-equal spinules. The abdominal appendages, now in the form of ill-defined papillae, are seen under the exoskeleton. Six pairs of segmental spines are clearly seen. The proximal and the distal abdominal spines are 10 and 30 μ long respectively. The antennule has two preaxial setae and its setation formula is 0–1–1–4–2–1–1–0.

The fifth nauplius, measuring 700 μ in total length, has a massive body. An elongated abdomen is very clearly segmented and the cirriform appendages, now in the form of well-defined papillae, are seen under the exoskeleton. The 240 μ abdominal process continues to be shorter than the caudal process. The spinulation pattern of the abdominal bulb remains unchanged from that of the earlier stage. The 25 μ long proximal abdominal spines move more laterally than 45 μ long distal abdominal spines. The former ones are short, stumpy with small pointed ends and the latter ones are long, slender with hyaline appearance. These two pairs of spines stay fairly apart from each other unlike those of *B.a. amphitrite* and *B.a. euamaryllis*. In the latter two species, these pairs approach closer at this and the following metanauplius stage. The spinulation on the stem portion of the abdominal process is considerably reduced and a ringlike pattern of the spinules on each ramus is largely disturbed. The terminal segment of the antennule becomes strikingly longer and the sub-terminal one becomes broad and bulbous. The setal pattern for the antennule is now 1–1–1–4–2–1–1–1.

The sixth larva is the largest metanauplius so far studied from the Indian Waters (Fig. 5). Measuring 780 μ in total length, it is about 200, 240, 280 and 300 μ longer than the metanauplii of *B. variegatus*, *B.a. amphitrite*, *I. cumingi* and *Ch. withersi* respectively. Its carapace, excluding the posterior carapace spines is 515 μ long and 410 μ wide. The carapace spines are 110 μ long. Each of the frontal horns is 70–75 μ long. The labrum is 160 μ long. A massive abdomen occupies most of the space under the carapace. Six pairs of segmental spines are present. Fully grown, the proximal and the distal abdominal spines are 30 and 50 μ long respectively. The spinules on the stem of the abdominal process become scarce and those on the rami lose their ringlike pattern so clearly observed at earlier naupliar stages. Many of these spinules crowd at the angle formed by the two rami. Each ramus is 95 μ long. The caudal process retains a few spines both on the body and on its base. The antennule now shows a setal pattern of 1–1–1–4–2–1–2–1. The metanauplius has three naupliar eyes.

TABLE III

Dimensions in microns of some cyprid features in six cirriped species from Bombay

Barnacle species	Carapace length	Carapace (l/w) ratio	Abdominal cirri	Caudal cirri	Antennular vacuum pad, diameter	Oil globules diameter
T. karandei	600	2.50	135	110	40	25
B. variegatus	615	2.23	120	100	*	10–35
B.a. amphitrite	510	2.15	*	*	50	15
Ch. withersi	480	2.23	95	85	35	*
Ch. malayensis	480	2.18	90	50	*	*
I. cumingi	565	2.22	120	*	30	25

* Not recorded.

The cyprid measuring 600 μ in carapace length is an extremely active larva (Fig. 6). A pair of massive antennules and the caudal appendages are constantly held out of the carapace. A delicate and transparent 240 μ wide carapace is boat-shaped and reveals encased soft body parts very clearly. Table III gives ratios of the length and the width (l/w) of the carapace of cyprids of various species examined in this laboratory. This ratio is the highest in respect of *T. karandei* cyprid. The presence of 180 μ long frontal filaments is a distinctive character of this cyprid. In no other cyprid these long filaments are observed. Each ramus of the abdominal appendages has four segments. The cirri on the abdominal appendages and the caudal appendage measure 130 and 110 μ respectively. A diameter of the antennular vacuum cup is 35 μ. The diameter of the oil globules present in the anterior half of the carapace is about 20–30 μ. Table III also gives the dimensions of the appendages of various cyprids examined in this laboratory.

Characters of diagnostic interest

The setation of antennule in *T. karandei* conforms to that of many tropical and temperate species examined by various workers. There are no preaxial setae at stages I and II and the first preaxial seta appears at stage III. There is addition of one each at stages IV and V. The fifth nauplius shows five post-axial setae and the sixth one has six of them. All the eight widely separated barnacle species examined in this laboratory show identical setal pattern. This character alone, therefore, is of no assistance in separating the larvae of one species from those of the other. However, since the number of setae is specific for each stage, it is possible to distinguish one stage from the other.

In *T. karandei* a true carapace with posterior carapace spines develops at stage IV. *T. karandei* larvae can be easily separated from the others by virtue of their long carapace spines (80 μ at stage IV and 110 μ at stage VI). No other species examined from Bombay waters has a carapace spine longer than 70 μ (Table IV). The carapace spine and spinulation is of considerable help in separating cirriped larvae encountered in Bombay waters. In the two chthamalid species studied, these posterior carapace spines are totally absent. In all other barnacles, the spines of varying lengths are present. A separation of the larvae is also possible on the basis of the presence or the absence of spinulation on the carapace border. In all the six nauplii of *B.a. euamaryllis* and *B. variegatus* the spinulation is present

TABLE IV

Comparison of naupliar carapace sculpture in eight cirriped species from Bombay

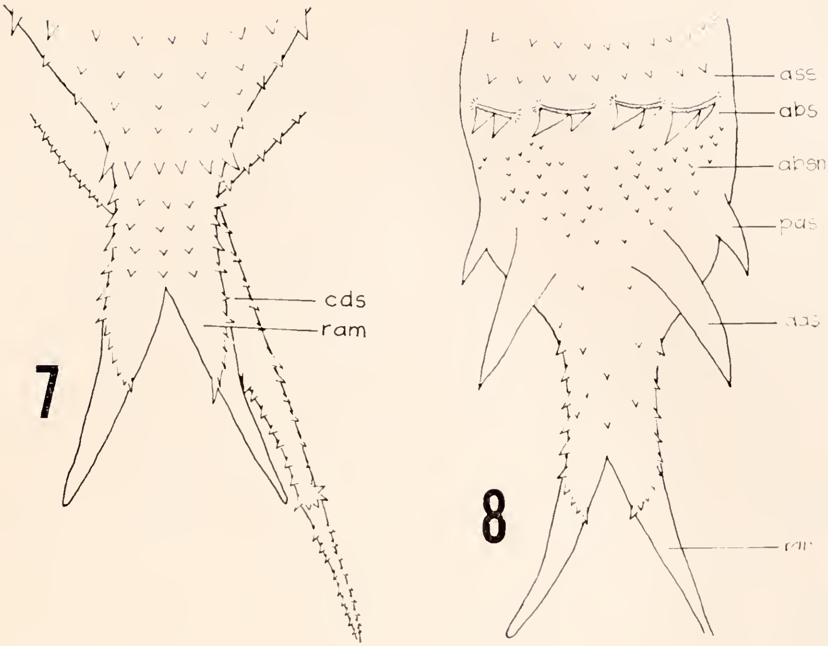
Barnacle species	Posterior carapace spines and lengths in μ			Carapace spinnules	Carapace spinnules
	IV nauplius	V nauplius	VI nauplius	False carapace, II and III nauplius	True carapace, IV, V and IV nauplius
T. karandei	80	85	110	Absent	Absent
B.a. amphitrite	35	35	15	One pointed spinule on either side	Absent
B. variegatus	35	50	45	Subequal spinules on entire border and on basal part of horn	Spinules persist
B.a. euamaryllis	50	70	*	Spinules on entire border	Difficult to locate or absent in VI nauplius
Balanus sp.	25	25	20	One spinule on either side	Absent
Ch. withersi	Absent	Absent	Absent	Very minute spinules, 7–8 on posterio-lateral sides bigger than the rest	Difficult to locate, or absent, V nauplius onward
Ch. malayensis	Absent	Absent	Absent	as above	May persist into VI nauplius
I. cumingi	50	35	35	Absent	Absent

* Not recorded.

along the entire border, including the area between two anterior horns. In the latter species, the spinulation extends little further over the bases of the anterior horns. In *Ch. malayensis* and *Ch. withersi*, seven or eight spinules located along the posterior-lateral border are bigger than those located on the lateral sides. All these spinules in these two species are however, difficult to locate when the larvae enter the fifth or the sixth stage. In *B.a. amphitrite* a single spinule is present on each of the lateral sides of the carapace. The carapace of *T. karandei* nauplii is spinuleless.

The caudal process in *T. karandei* is always longer than the abdominal process. A relative difference in the lengths of these two processes is not constant at any naupliar stages and this feature therefore is of no help in separating various *T. karandei* larvae. The caudal processes of the metanauplii of *T. karandei, B.a. euamaryllis, B.a. amphitrite, B. variegatus, Ch. withersi* and *Ch. malayensis* measure 270, 200, 160, 115, 65 and 60 microns respectively. These lengths, however, vary considerably depending upon the growth conditions of the larvae. In the pedunculate species *I. cumingi,* the caudal process is totally lost at fourth naupliar stage.

An abdominal process is comprised of two pairs of abdominal spines, a median spine, a stem and its two rami. As with many other barnacles, the spines and spinulation on the abdominal process, their number, and their sequence of emergence or disappearance are of diagnostic value in *T. karandei.* A distal pair of abdominal spines appears at stage II and a proximal pair appears at stage III. These two pairs persist into the metanauplius stage. A similar situation is also noted in



FIGURES 7–8. Abdominal processes of the second and fourth naupliar stages of *Tetraclitella karandei;* ass, abdominal segmental spinules; abs, abdominal spines; absn, abdominal bulb spinules; cds, caudal spine; pas, proximal abdominal spines; das, distal abdominal spines; ram, ramus.

B.a. amphitrite and *B.a. euamaryllis.* In *B. variegatus* however, both the proximal and the distal pairs appear at second nauplius stage.

A single median abdominal spine between the members of the proximal pair of abdominal spines is absent in *T. karandei* fourth nauplius. This spine is present in *B.a. communis* (Pillai, 1958), *B. variegatus* (Karande, 1973b; in press), *B.a. euamaryllis* and in a few other balanid species like *B. balanus* (Barnes and Costlow, 1961) and *B. hesperius* (Barnes and Barnes, 1959). An absence of a median abdominal spine is a distinctive diagnostic character that helps to separate the fourth *T. karandei* nauplius from many other similar nauplii of balanids.

The teeth on the labrum, their presence and pattern or their absence, are useful in separating various cirriped larvae encountered in Bombay waters. The second nauplius of *T. karandei* shows a pecten of very minute teeth on each of the lateral lobes of its trilobed labrum. In the subsequent stages, however, these teeth are lost. The labrum in these later nauplii therefore appears similar to those of *B.a. amphitrite* and *B. variegatus* and lacks teeth. In *B.a. euamaryllis,* two or three sub-equal teeth are present on the middle lobe of the trilobed labrum. In *I. cumingi,* the labrum has an indented free margin that bears four to six teeth. These teeth are retained until the end of the metanauplius stage. The second nauplii of *Ch. withersi* and *Ch. malayensis* bear a series of 7–8 teeth. All other stages of these species, however, bear only two prominent teeth.

DISCUSSION

In general, the development of cirriped larvae is very uniform (Barnes and Barnes, 1959). The development of tetraclitellan barnacles, as judged by the present study on *T. karandei,* is no exception to this. The similarity between balanid and tetraclitellan metamorphosis, in fact, is so close that in the absence of detailed morphological descriptions, nauplii of these barnacles are likely to be mistaken for each other. For instance, the hitherto undescribed larvae of *T. karandei* could have been easily mistaken for the more common balanid larvae. This, however, is not true of the chthamalid and the pedunculate species encountered in Bombay waters. These barnacles follow the general cirriped metamorphosis plan but their identity as chthamalid or pedunculate larvae can be readily established on the basis of the presence or the absence of certain important morphological characters.

The present description of *T. karandei* larvae will permit their ready separation from the other cirripid larvae encountered in Bombay waters. Some of the salient features of the nauplii of this species are the larger dimensions, smooth carapace border, large posterior carapace spines, the sculpturing of the rami and a distinctive spinulation of the abdominal bulb. The nauplii of this species, because of their larger sizes cannot be mistaken for those of the chthamalid and the pedunculate species. They also cannot be confused with the larvae of *B. variegatus* or *B.a. euamaryllis* both of which have highly spinulated carapace borders. These nauplii have some resemblance with comparatively smaller sized larvae of *B.a. amphitrite.* Notwithstanding, the second and the third nauplii can be separated from the corresponding larvae of *B.a. amphitrite,* because in the latter the carapace bears a small but distinct spinule on its either side. The fourth, the fifth and the sixth nauplii of *T. karandei* can be separated from the corresponding larvae of *B.a. amphitrite,* because in the latter species the posterior carapace spines are strikingly shorter than those of the former one.

The larvae of *T. karandei,* like those of *B.a. amphitrite, B. variegatus, Ch. malayensis, Ch. withersi* and *I. cumingi* examined here, take generally eleven or twelve days to reach the cypris stage. The first nauplius of *B.a. amphitrite* may at times reach the cypris stage within five days, which is the shortest recorded larval cycle for any operculate barnacle species.

I am thankful to Shri C. P. De, Director of this laboratory for his continued interest and encouragement in this work. I thank Shri K. B. Menon who supplied me with gravid adults of *Balanus amaryllis euamaryllis.* Thanks are also due to Shri M. K. Thomas who helped me maintain algal cultures in this laboratory.

SUMMARY

Tetraclitella karandei and *Tetraclita purpurascens* are two intertidal species encountered along the Bombay coast of India. The larval stages of *T. karandei* from the first nauplius through cyprid larva are described and illustrated. The naupliar characters such as setation of antennules, carapace sculpture, and the occurrence of the abdominal bulb and caudal process have been critically examined. Their usefulness as characters of diagnostic value is discussed. The naupliar stages of this

species exhibit features, notably the spinulation of the carapace and an abdominal process, which permit their ready separation from the nauplii of all other local species. The present study strengthens the view that, in general, development amongst cirriped nauplii follows a uniform pattern.

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MOVEMENT OF PIGMENT GRANULES WITHIN MELANOPHORES OF AN ISOLATED FISH SCALE. EFFECTS OF CYTOCHALASIN B ON MELANOPHORES

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Pigment granules within melanophores of various fish have been found to disperse in a physiological saline solution and to concentrate in M/7.5 (isotonic) KCl or adrenalin-physiological saline solutions. Such changes result from movement of the intracellular pigment granules (Spaeth, 1913; Matthews, 1931). There have been many reports by a considerable number of investigators on the intracellular movement of these granules, but the exact nature of the mechanism involved still lacks sufficient clarification (Ballowitz, 1914; Spaeth, 1913, 1916; Matthews, 1931; Biedermann, 1926; Marsland, 1944; Kamada and Kinosita, 1944; Kinosita, 1953, 1963; Bikle, Tilney and Porter, 1966; Wikswo and Novales, 1969, 1972; Ohta, 1971).

Lately, detailed electron microscopic studies on the fine structure of melanophores have been reported which cite the presence of many microtubules, microfilaments, endoplasmic reticulum and the like (Bikle, Tilney and Porter, 1966; Green, 1968). Wikswo and Novales (1969, 1972) have investigated the relationships between microtubules and movement of pigment granules, using colchicine for its so-called effect on intracellular microtubules (Borisy and Taylor, 1967a, 1967b; Shelanski and Taylor, 1967). The same authors indicate that colchicine inhibits concentration of the granules so as to foster their dispersion. Results of electron microscopy, moreover, revealed that microtubules in melanophores decrease significantly with colchicine treatment while microfilaments show relative increase. Malawista (1971), Novales and Novales (1972), McGuire and Moellmann (1972), and McGuire *et al.* (1972), have investigated cytochalasin B, known for its action upon microfilaments, for its effects upon the skin melanophore of the frog; however, there are no reports as yet on fish melanophore.

The present study aimed to investigate the relationships between various intracellular structures of melanophores and the movement of pigment granules. To achieve this, cytochalasin B was employed, and the effects on pigment granules movement of fish melanophore were investigated.

MATERIAL AND METHODS

The materials were melanophores from the isolated scale of *Oryzias latipes* (the wild type) which inhabit waters in and around the cities of Okazaki and Seto in Japan. Employing a pincette with sharp tips, the scale was carefully removed from the dorsoventral part of the fish and fixed, epidermis side down, to a glass coverslip mounted on a glass trough. Following isolation in this manner, the scale was kept immersed in a physiological saline solution, and after making certain that the granules had completely concentrated in an M/7.5 KCl solution, the test scale was again put into the physiological saline solution for 10 min. Then, the melanophores were subjected to cytochalasin B (I. C. I., England).

For the physiological saline solution, an M/7.5 balanced solution according to Yamamoto (1939) was used. The cytochalasin B concentration was 1 μg/ml and 10 μg/ml-0.1% DMSO (dimethylsulfoxide)-physiological saline solution (pH adjusted to 7.2 by $NaHCO_3$). For the solution in which to concentrate the pigment granules, an M/7.5 KCl (pH 7.2 by $KHCO_3$) and 10^{-6} M adrenalin-physiological saline solution were employed. The experiments were run at room temperature, between 20–23° C.

Melanophore variations were measured photoelectrically (Fujii, 1959). Light was thrown on the photocell attached to one lens of the binocular microscope. Then, the photoelectric current was recorded (San'ei sokki, type 8C12-1119 test recorder) after amplification by an amplifier (San'ei sokki, type 1205C). Microscopic observations of melanophores were performed with another lens.

Denervated melanophores were obtained by the operation performed upon fish scales as described in the previous paper (Ohta, 1972). The scale was first carefully isolated from the fish with a pincette under a stereoscopic microscope, immediately returned to the original position, and the fish was kept for over 18 hr at 28° C against a black container. Then the scale was removed once more and put in an M/7.5 (isotonic) KCl solution in which it exhibited no concentration whatsoever.

RESULTS

Response of innervated melanophores to cytochalasin B

The innervated melanophores were subjected to cytochalasin B action at 1 μg/ml and 10 μg/ml concentrations, continuously for 60 min. The melanophores thus sustained a state of dispersion. With the above photoelectric method, a slight concentration response with recorded several minutes after drug action, but a constant state held thereafter. This was opposed to control (physiological saline solution) in which a constant state was maintained. Under microscopic observation, however, no concentration of pigment granules toward the centrosphere was found to be involved. Rather, there was movement out to the branch extremities so that pigment granules in the centrosphere were markedly reduced in number (Figs. 2A, 2B). This was the super-dispersion phenomenon, and with it was observed an extremely active Brownian movement of pigment granules. Almost no effect on melanophores was noticed from the 0.1% DMSO used to dissolve the cytochalasin B.

Next, after subjection to respective cytochalasin B concentrations for 30 and 60 min, concentration time of granules in M/7.5 (isotonic) KCl and then dispersing time in physiological saline solutions were observed. The results are indicated in Table I and II. At 30 min and the concentration induced by M/7.5 (isotonic) KCl solution thereafter, less time was required for pigment granules to attain full concentration as compared to control. However, this was entirely in terms of cytochalasin B concentration. There was little or no difference from controls after treatment with 0.1% DMSO. Five minutes following complete concentration in M/7.5 (isotonic) KCl solution and changing the external medium to a physiological saline one, the time for pigment granules to begin and complete dispersion was observed to be much shorter than in control (Table I, II).

TABLE I

Response of innervated melanophores to M/7.5 (isotonic) KCl and physiological saline solution after treatment with cytochalasin B for 30 minutes. Abbreviations used are; T-1: Length of time between exchange of external medium and start of pigment concentration (min); T-2: Length of time between exchange of external medium and complete pigment concentration (min); T-3: Length of time between exchange of external medium and start of pigment dispersion (min); T-4: Length of time between exchange of external medium and complete pigment dispersion (min).

	No. of tests	M/7.5 KCl		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	11	0.9 ± 0.33	1.8 ± 0.33	3.0 ± 0.66	6.4 ± 1.47
1 μg/ml Cytochalasin B	11	0.6 ± 0.30	1.2 ± 0.39	1.5 ± 0.67	4.2 ± 0.98
10 μg/ml Cytochalasin B	10	0.2 ± 0.17	1.1 ± 0.27	0.5 ± 0.27	2.2 ± 0.49
0.1% DMSO	11	0.8 ± 0.30	1.7 ± 0.41	2.8 ± 0.60	5.9 ± 1.17

With 60-min exposure to the drug, there was more pronounced granule concentration or dispersion, and obviously both outstripped controls. The rate was much more accelerated than with 30-min exposure. Microscopic observation of pigment granule dispersion in the physiological saline solution showed that these granules tended to become very scarce in the centrosphere, the movement being out toward the branch extremities as dispersion occurred.

Response of denervated melanophores to cytochalasin B

Denervated melanophores were subjected to 60 min of 10 μg/ml cytochalasin B Results nearly paralleled those with the innervated melanophore. Both super-dispersion and an active Brownian movement were also observed.

Next, after 30- and 60-min exposure to 10 μg/ml cytochalasin B, the melanophores were subjected to 5 min of 10^{-6} M adrenalin followed by change of external medium to the physiological saline one. Then the time for pigment granules to con-

TABLE II

Response of innervated melanophores to M/7.5 (isotonic) KCl and physiological saline solution after treatment with cytochalasin B for 60 minutes; Abbreviations as in Table I

	No. of tests	M/7.5 KCl		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	9	1.2 ± 0.43	2.1 ± 0.51	3.6 ± 0.61	8.3 ± 1.00
1 μg/ml Cytochalasin B	9	0.4 ± 0.24	1.3 ± 0.43	2.4 ± 0.92	5.7 ± 2.19
10 μg/ml Cytochalasin B	9	0.1 ± 0.08	0.9 ± 0.18	0.5 ± 0.27	1.8 ± 0.51
0.1% DMSO	9	1.3 ± 0.56	2.2 ± 0.64	3.7 ± 0.96	7.5 ± 2.09

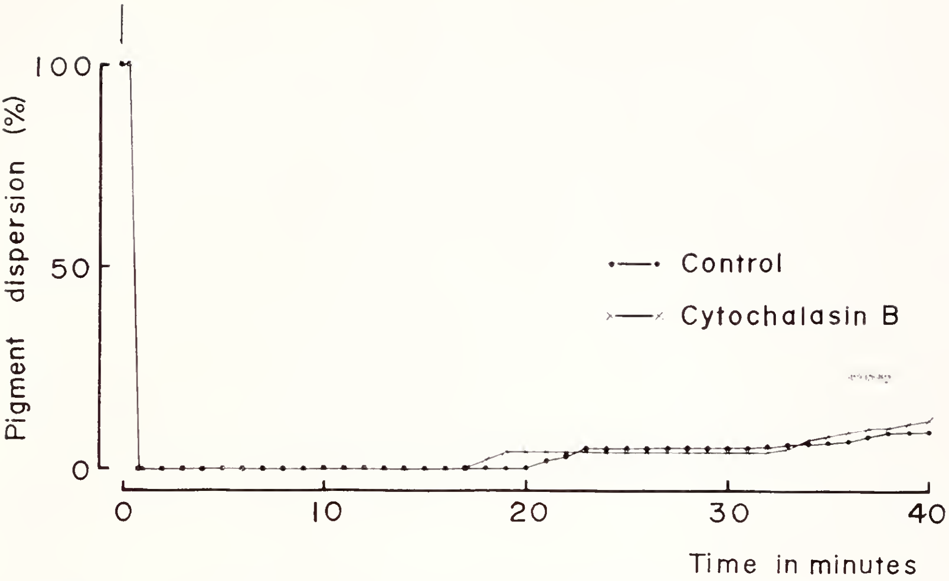

FIGURE 1. An example of cytochalasin B response of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution. Horizontal indicates time after the solution was applied (arrow), room temperature, 21° C.

centrate and disperse was measured. Table III and IV provide the results. In the case of denervated melanophores exposed to adrenalin, the time until pigment granules began to concentrate and then reach complete concentration was extremely fast: at 30-min exposure, 0.1 min for the former, 0.9 min for the latter as against 0.6 and 2.3 min respectively with controls. Again, with dispersion, against the 17.4 min it took controls to attain 50% dispersion level, drug-treated pigment granules took 8.3 min, only half the time.

There was no special difference of time in concentration between 60-min or 30-min treatment, but in dispersion of pigment granules the dispersal process for 60 min was much faster than for 30-min exposure (Table III, IV).

TABLE III

Response of denervated melanophores to $10^{-6}M$ adrenalin and physiological saline solution after treatment with cytochalasin B for 30 minutes; abbreviations as in Table I except T-4: length of time between exchange of external medium and 50% pigment dispersion (min)

	No. of tests	Adrenalin		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	10	0.6 ± 0.25	2.3 ± 0.83	5.9 ± 2.16	17.4 ± 3.50
10 μg/ml Cytochalasin B	10	0.1 ± 0.08	0.9 ± 0.09	2.0 ± 0.68	8.3 ± 1.60

TABLE IV

Response of denervated melanophores to 10^{-6} M adrenalin and physiological saline solution after treatment with cytochalasin B for 60 minutes; abbreviations as in Table I except T-4: length of time between exchange of external medium and 50% pigment dispersion (min)

	No. of tests	Adrenalin		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	9	0.5 ± 0.14	2.2 ± 0.80	5.7 ± 2.71	16.3 ± 2.65
10 μg/ml Cytochalasin B	10	0.1 ± 0.12	1.0 ± 0.32	1.3 ± 0.98	4.3 ± 1.91

Cytochalasin B response of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution

Cytochalasin B action at 10 μg/ml was investigated with innervated melanophores fully concentrated in M/7.5 (isotonic) KCl solution and the results are given in Figures 1, 2C and 2D, and Table V. In M/7.5 (isotonic) KCl solution guanophores co-existing with the melanophores behave quite oppositely, but since their response is slow, photoelectric recording noted gradual dispersion often maximum concentration of pigment granules within melanophores had been obtained. However, once the guanophores were fully dispersed, an almost uniform response was recorded.

With cytochalasin B action, 3 of 10 samples showed dispersion degree greater than controls at 40 min (30%, 42%, 65%, respectively). At 10 or 20 min there was no great variation from controls, but at 30 or 40 min the mean dispersion rate nearly doubled. Nevertheless, this mean value difference was not statistically significant at 30 or 40 min.

Pigment granules dispersed very rapidly following exposure to cytochalasin B and change of external medium to a physiological saline one. In this situation nearly all pigment granules left the centrosphere, dispersing out to the branch extremities (Figs. 2E and 2F), a phenomenon often seen with diluted NaCl solution as reported earlier (Ohta, 1972).

TABLE V

Response (% of pigment granule dispersion at each time) of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution to cytochalasin B

	No. of tests	After	After	After	After
		10 min	20 min	30 min	40 min
Control	10	1.6 ± 3.90	6.5 ± 6.66	9.5 ± 8.15	10.5 ± 9.92
10 μg/ml Cytochalasin B	10	0.8 ± 1.66	7.3 ± 9.63	15.0 ± 14.73	20.7 ± 19.64

DISCUSSION

In 1967, Carter reported the interesting finding that, when cytochalasin B derived from mould metabolite separation acted upon fibroblasts, nuclear division remained normal but cell division was blocked. Subsequently, Schroeder (1969) found that, from an electron microscopic picture of the *Arbacia* egg treated with cytochalasin B, the contractile filaments which develop in the region of the cleavage furrow are notably absent, but no changes in the spindle-shaped microtubules appear. Follow-up studies have since been conducted not only on them but with Hela cells (Schroeder, 1970), lymphocytes (Ridler and Smith, 1968) and the like (Wessells *et al.*, 1971), and these have supported Carter's observations. Cytochalasin B is claimed to inhibit dispersion of pigment granules in frog skin melanophore (Malawista, 1971; Novales and Novales, 1972; McGuire and Moellmann, 1972; McGuire *et al.*, 1972). According to McGuire and Moellmann (1972) and McGuire *et al.* (1972), cytochalasin B even causes concentration of pigment granules though a dispersing agent is present, but does this while markedly reducing the number of microfilaments. Magun (1973), however, failed to find results as striking, so there would seem to be some disagreement about the validity of this particular effect of cytochalasin B.

On the other hand, electron microscopic observations have shown the presence of microtubules, microfilaments and endoplasmic reticulum in melanophores of fish (Bikle, *et al.*, 1966; Green 1968). Wikswo and Novales (1969, 1972), using colchicine, have researched its effect on melanophores of *Fundulus*. According to their physiological results, colchicine treatment of this type of melanophore leads to inhibition of pigment granule concentration and, conversely, promotion of dispersion. Upon electron microscopic observation, the microtubules within the melanophores were recognized to decrease and the microfilaments to increase. From these results, the same authors suggested that decrease of the microtubules may be related to inhibition of pigment granule concentration and the increase of the microfilaments to promotion of pigment granule dispersion.

Results of the present experiments indicate that treatment of melanophores with cytochalasin B, which has the earlier-mentioned effects, has a definite accelerative effect on movement—concentration and dispersion—of pigment granules, and that this effect is dependent upon time and drug concentration. This present observation, namely that cytochalasin B enhances the rate of response to adrenalin, is similar to that pointed out by McGuire and Moellmann (1972) and McGuire *et al.* (1972). Nevertheless, with melanophores in which pigment granules are in a state of dispersion, the use of cytochalasin B obtains no concentration at all. As evident from Figures 2A and 2B, the pigment granules are almost inexistent in the centrosphere, having dispersed to the branches. Lyerla and Novales (1972), in their observations and report on frog skin melanophore, have noted a similar effect of cytochalasin B as peculiar to the melanophore form. But with cytochalasin B, on the other hand, granules completely concentrated in M/7.5 (isotonic) KCl solution evidenced a somewhat scattered effect: after 30 or 40 min, dispersion levels greatly exceeded controls, but full dispersion was not attained.

Taken from the standpoint of cytochalasin B action, it is difficult to ascribe any direct kinetic capacity to the microfilaments when concentration or dispersion

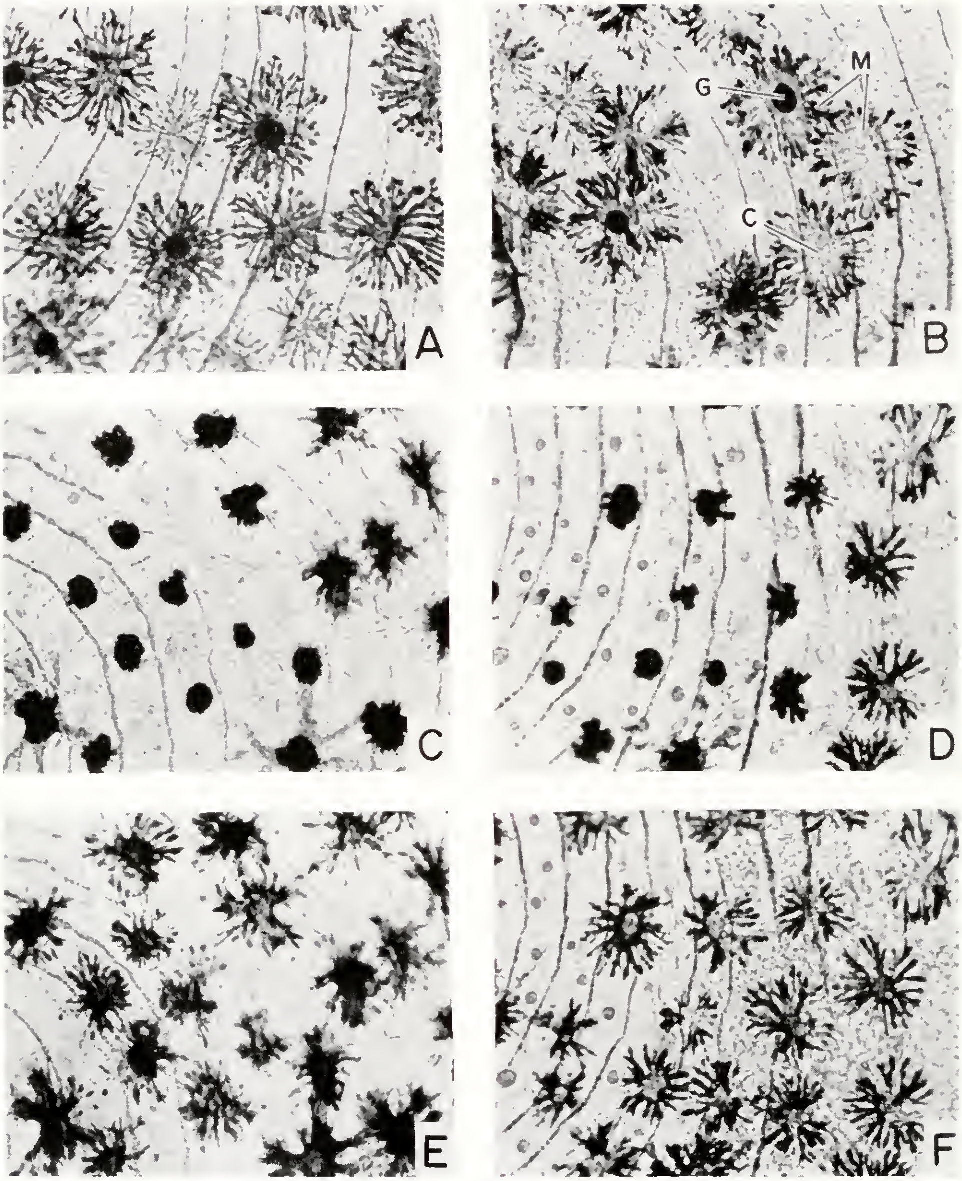

FIGURE 2. Photos of innervated melanophores treated with cytochalasin B; A, Control (in physiological saline solution; B, Innervated melanophore treated with 10 μg/ml cytochalasin B-physiol for 30 minutes; C, Control (in M/7.5 KCl solution); D, Innervated melanophore treated with 10 μg/ml cytochalasin B-M/7.5 KCl for 40 minutes; E, Control (dispersed condition of innervated melanophore in photo C by physiological saline solution); F, Dispersed condition of innervated melanophore in photo D by physiological saline solution; × 600. Symbols used are: M, Melanophore; G, Guanophore; C, Centrosphere.

movements take place with the granules inside melanophores. Instead, these pigment granules would appear to be inhibited in the course of normal movement by microfilaments. Further morphological observations by electron microscope are ncessary, particularly upon microfilaments in the melanophore before and after use of cytochalasin B. Moreover, more detailed research from various aspects is required on the relations between pigment granule movement and elments of intracellular structure.

Summary

1. Employing melanophores from an isolated medaka (*Oryzias latipes*) scale, this study sought to clarify the mechanism of pigment granule movement by investigating cytochalasin B action.

2. Observations were made upon 1 μg/ml and 10 μg/ml cytochalasin B innervated melanophores at 30 and 60 min, respectively, and followed by application of M/7.5 (isotonic) KCl, then physiological saline solution.

3. Results showed a marked acceleration of concentration and dispersion in pigment granules through cytochalasin B compared to control, and this effect was dependent upon time and drug concentration.

4. Approximately the same results obtained with denervated melanophores when adrenalin was employed instead of M/7.5(isotonic) KCl solution to concentrate pigment granules. The 0.1% DMSO (dimethysulfoxide) used to dissolve the cytochalasin B was not found to affect movement of pigment granules.

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FACTORS AFFECTING GERMINATION, GROWTH, AND DISTRIBUTION OF THE FRESHWATER SPONGE, *SPONGILLA FRAGILIS* LEIDY (PORIFERA)

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Various field surveys have been conducted to determine which physicochemical parameters affect the distribution of freshwater sponges (Old, 1932; Jewell, 1935, 1939; Cheatum and Harris, 1953; Penney, 1954). Old (1932) concluded that perhaps sponge distribution is influenced by the interaction of environmental factors rather than a single factor, while Jewell (1939) emphasized the role of calcium. Wurtz (1950) summarized the range of values for various parameters affecting individual species distribution. The general biology of the freshwater sponges has been reviewed by Hyman (1940) and Pennak (1953), although there have been few ecological studies dealing with them. No annual survey of a system has been attempted although seasonal fluctuations of important parameters may affect species distribution.

More recent investigations have been devoted to rearing freshwater sponges from gemmules in the laboratory (Rasmont, 1961) to elucidate the physiology of gemmulation and germination, and, more generally, the process of dormancy (Rozenfeld, 1970, 1971; Rasmont, 1962, 1963, 1970; Rasmont and Schmidt, 1967).

This study was initiated to determine (1) the effects of physicochemical factors on sponge gemmule germination and early sponge growth in the laboratory, and (2) which of these factors may be limiting during the year to affect species distribution.

MATERIALS AND METHODS

The species studied

The freshwater sponge *Spongilla fragilis* inhabits a narrow range in Rapid Run, a mountain stream within R. B. Winter State Park, Union County, Pennsylvania. It is found immediately behind Halfway Dam and in the pool below it attached to smooth concrete surfaces, on submerged twigs, within moss mats, and on the undersides of rocks. It occupies the facing of large rocks at the end of the spillway below the pool and it may be found beyond the spillway for 0.6 mile on the undersides of rocks within the moderately flowing stream. It is not found in the remaining 9 mile course, nor in the stream anywhere above the dam.

Exposed sponges tend to be gray in color; although green sponges (indicating the presence of symbiotic algae) have been reported, none were found during 1972. A white color is characteristic of sponges found under stones.

Germination experiments

Gemmules of *S. fragilis* were collected below the spillway in April, 1972 (one month before germination) by scraping brown mats of them from the undersides of rocks. These were transferred to a gallon jar filled with distilled water and refrigerated at 2° C until utilized. Identification was made from permanent slides of sponge and gemmule spicules prepared by nitric acid digestion (Pennak, 1953).

Prior to investigation the gemmules were sterilized in a dilute hydrogen peroxide wash and rinsed in distilled water (Rasmont, 1961). Intact gemmules in groups of one, two, and three were selected to facilitate germination and size determinations. These were transferred with surgical forceps to 60 × 15 mm plastic petri dishes containing the various test solutions. Inspection was made to ascertain that no physical damage occurred during transfer.

A threshold temperature for germination was determined by conducting experiments in the dark using three temperatures: $6 \pm 1.0°$ C, $8 \pm 0.5°$ C, and $10 \pm 0.5°$ C. Unless indicated otherwise, germination experiments were conducted at a constant $10 \pm 0.5°$ C, representing an approximate summer mean for Rapid Run. An appliance timer regulated a 60-watt incandescent bulb for a 12-hour-light: 12-hour-dark cycle; dishes were covered with foil for the dark condition.

Measurements were made after 17 days of incubation. Germination was determined by noting the presence of cellular material at the micropyle. Size of the germinated sponge was measured with a Whipple Disc. General morphology was noted in all cases, since the mere presence of cellular material gives no indication of differentiation.

An artificial medium similar in major ion concentration to Rapid Run water was devised to insure consistent conditions in all experiments. Anions and cations were chosen according to Rasmont (1961). Cation concentrations were: magnesium ($MgSO_4 \cdot 7H_2O$) and calcium ($CaCO_3$) – 2 mg/l; sodium ($Na_2SiO_3 \cdot 9H_2O$) and potassium (KCl) – 0.5 mg/l. Suitability of Rapid Run water, Rapid Run Medium (RR Med), and double-distilled water (× 2) as growth media was tested under light-dark and dark conditions in 18 ml of solution. Dilutions were made of RR Med to establish minimum concentration requirements for normal growth, compared to RR Med. The importance of the individual compounds of RR Med was tested by omitting each from the medium, and also by adding each in normal concentration to distilled water; these were grown under 12:12, light-dark, conditions. The importance of magnesium, sulfate, calcium, and carbonate was tested by omitting each in turn from RR Med (replacing cations with K^+, and anions with Cl^-). Cations combined with Cl^- and anions with K^+ were also added to distilled water and tested.

Experimental design was as follows: for determining the effect of light and growth media on germination, 5 dishes with 50 gemmules each were used; 4 dishes with 50 gemmules each were used in all remaining experiments.

Statistical methods

Data for per cent germination and size were analyzed using a standard one-way ANOVA table and compared for Least Significance Difference at the 5% and 1% levels.

Field study

A study of a number of environmental parameters (Jewell, 1935) was conducted from March, 1972, to March, 1973, to determine which of them may influence (or limit) distribution, and to see what effect the dam and lake may have on water quality and hence distribution of sponges.

Sample sites were chosen according to sponge distribution: #1-Rapid Run incurrent to Halfway Dam; #2-immediately downstream from the spillway; #3-approximately 3.7 miles downstream from the spillway.

Temperature and pH were measured on location using a standard mercury thermometer and portable pH meter, respectively. Water samples were taken in one liter polyethylene bottles. All other parameters except silica were tested within one hour in the laboratory. Dissolved oxygen was not determined since turbulence would probably prevent it from being limiting.

Alkalinity was tested by a potentiometric method for low alkalinity (American Public Health Association, 1971). Calcium was measured titrimetrically (EDTA Method, American Public Health Association, 1971). Filtered and unfiltered samples gave identical values for silica, measured colorimetrically (Reactive Silicate, Golterman, 1969). Ionic concentration was determined with a Hach conductivity meter. Apparent color was measured with a Hach portable colorimeter using a #5543 filter.

Sampling was postponed at least two days following rain to circumvent dilution effects.

RESULTS

Gemmules used in the experiments had a mean diameter of 350 $\mu \pm 61$ μ. No correlation was noted between size and the ability of the gemmule to germinate and grow under various light conditions in similar media.

The results of experiments comparing growth media and light versus light-dark conditions are summarized in Figure 1. Sizes were not compared in experiments conducted under dark conditions because fusion of individual sponges had decreased the sample number.

RR Med appeared to be comparable to stream water with regard to germination and growth, regardless of light condition. The two media also produced morphologically similar organisms complete with oscula, canals, and spicule skeletons. Double-distilled water (× 2) produced significantly fewer (1% level) sponges in L:D which were considerably smaller and appeared undifferentiated. Germination in distilled water in total dark was significantly less (1% level) than L:D.

The experiment with distilled water and total dark was repeated to verify the initial results. There was no germination after 18 days. The foil was removed from containers and incubation was continued under light-dark conditions. After 13 additional days, germination was 16%.

All experiments testing the effect of temperature (see Figure 2) were conducted in total dark, except when exposed to light for a few minutes for counting purposes. Some temperature fluctuation may have occurred then also, but presumably not enough to affect results. A threshold appears to exist between 6° and 8° C; no germination was observed at the lower temperature following incubation for 30 days. An increase in temperature up to 20° C resulted in decreased germination time. Since results from 16° C and 20° C were similar, it was concluded that 16° C is a suitable temperature for conducting germination experiments.

Diluting RR Med to 1/100 (RR Med/100) appeared to have no effect on the

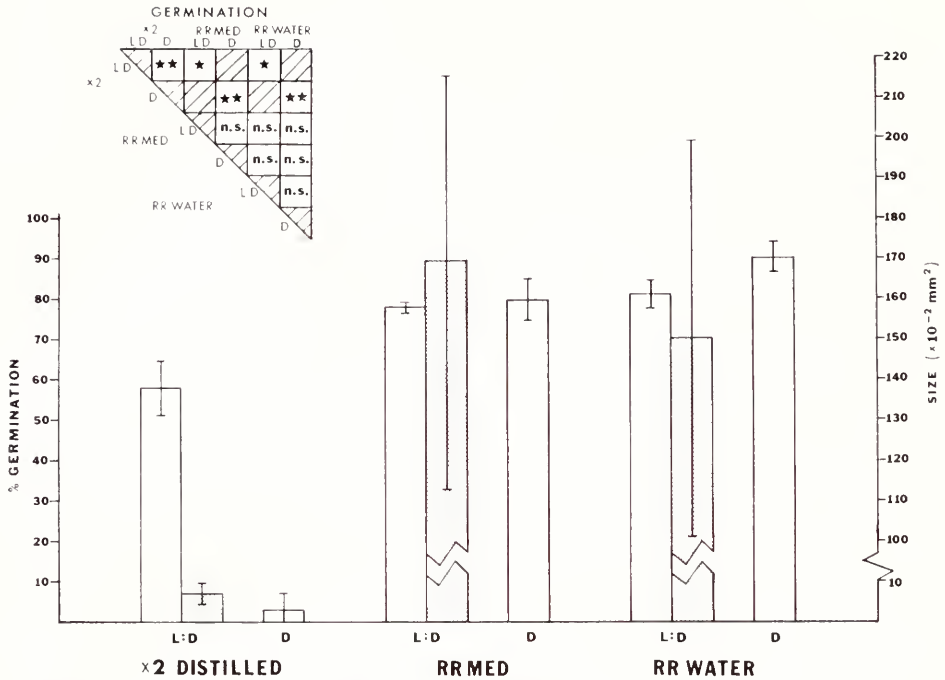

FIGURE 1. Response of gemmules of *Spongilla fragilis* to various media and light conditions. Solid bars represent per cent germination, and shaded bars represent size. Vertical lines indicate standard deviation (★★ = signficance at the 1% level; ★ = 5% level; n.s. = not significant).

rate of germination (see Figure 3). Germination decreased significantly (1% level) at RR Med/500, a value closely approximating germination in distilled water. Although RR Med 1000 supported greater germination than RR Med/500, the difference in response between the two conditions was not significant.

The effective tolerance of the organism to low concentrations of inorganic material is better illustrated by growth response. Sponges grown in RR Med 10 were similar to RR Med sponges in size and general morphology, complete with spicules (except that the number of spicules in RR Med/10 was smaller). In RR Med/100 and more dilute media, growth was significantly (1% level) retarded; the sponges were amorphous although some pores were present in RR Med/100; spicules were absent in all cases.

Clusters of spicules which remain after nitric acid digestion would seem to indicate the establishment of a skeletal network. These were noted in dilutions between RR Med and RR Med/10; however, spicule distribution was somewhat random in the dilution.

It may be concluded from this phase of the study that "normal" sponge growth in the laboratory is supported by concentrations of 0.2 mg/l Ca and Mg, 0.05 mg/l K and Na, and 0.06 mg/l SiO_2.

Exclusion of the individual compounds from RR Med did not affect rate of germination. However, growth and morphology were greatly affected (see Figures 4 and 5).

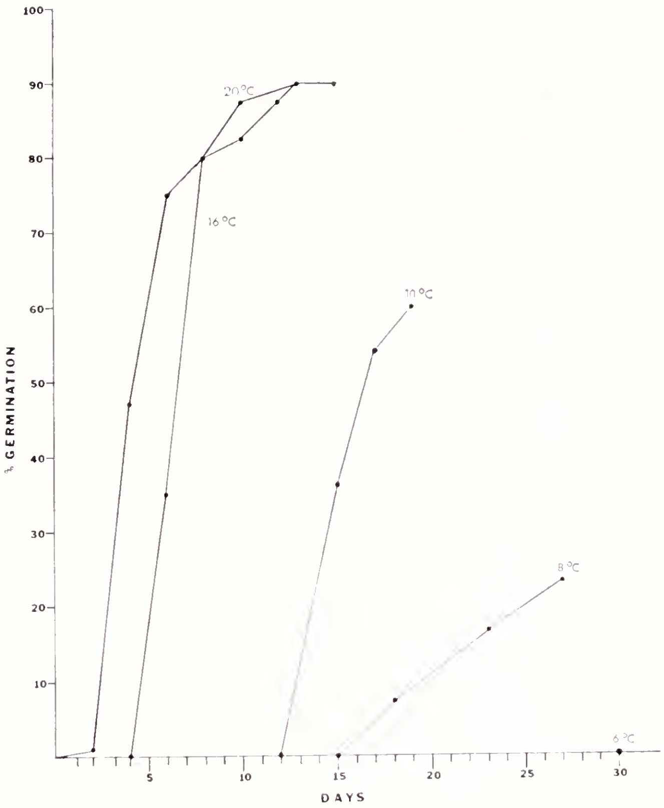

FIGURE 2. Germination response of *S. fragilis* gemmules to temperature.

RR Med lacking KCl and Na_2SiO_3 resulted in sponges of similar size; spicules were absent in the solution lacking SiO_2, but other features appeared typical. These groups differed significantly (1% level) in size from those lacking $CaCO_3$ and $MgSO_4$.

Sponges in RR Med w/o $CaCO_3$ showed a tendency to be smaller than those in RR Med w/o $MgSO_4$. Morphologically these sponges were undifferentiated granular masses with no spicules; the major difference was that the cells in RR Med w/o $CaCO_3$ remained clustered around the gemmules, while those in RR Med w/o $MgSO_4$ emigrated.

Omitting $CO_3^{=}$, Ca^{++}, $SO_4^{=}$, Mg^{++} in turn from RR Med produced dubious results with regard to germination. However, differences in growth were spectacular (see Figure 6). RR Med lacking either Ca or Mg produced significantly (5% level) smaller sponges than RR Med lacking SO_4 or CO_3. Although all conditions supported spicule production, only random spicules were found with Ca or Mg lacking. Negligible differentiation (although some sponges in RR Med w/o Mg had oscula) resulted although a regular skeleton was formed with both cations present. Presence of CO_3 and SO_4 (or perhaps an increased concentration of Cl) supported spicules in conjunction with either cation.

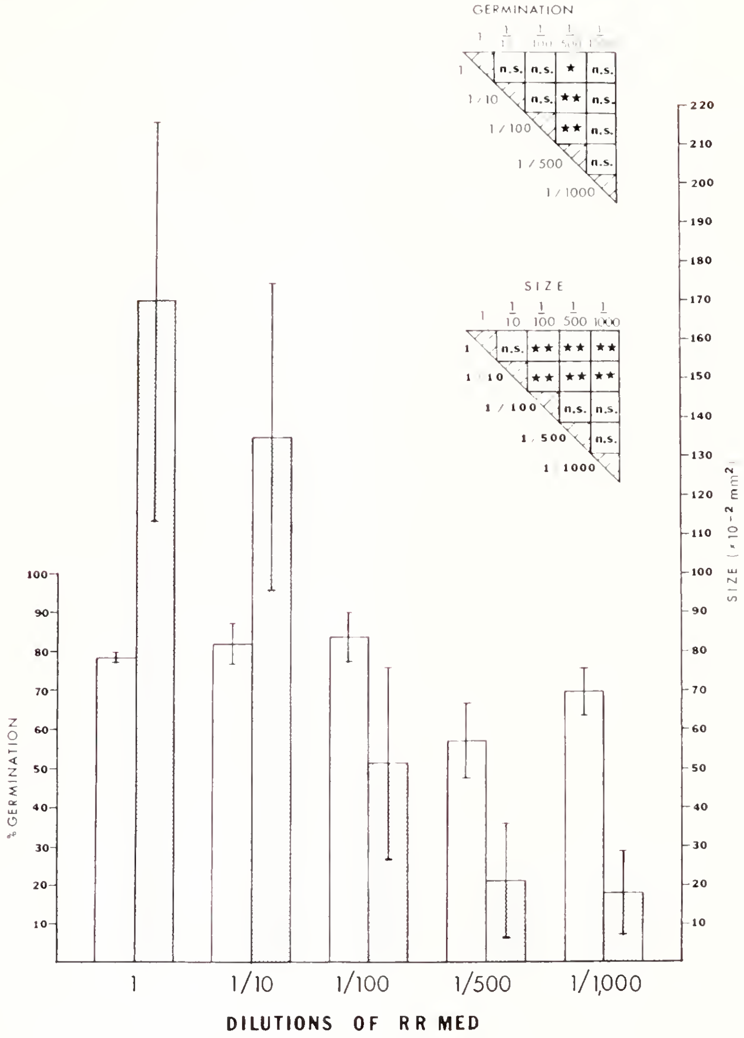

FIGURE 3. Repsonse of *S. fragilis* gemmules to dilution of basic Rapid Run Medium (RR Med); conventions as in Figure 1.

Adding the individual compounds to distilled water provided inconclusive results with respect to germination. Although $CaCO_3$ + distilled water contained significantly (1% level) larger sponges than the other compounds, they were undifferentiated and doubtfully functional.

Individual cations (as chlorides) and divalent anions (with K^+) were tested in distilled water. Only KCl resulted in a lower frequency of germination. Again Ca promoted the greatest growth of undifferentiated sponge.

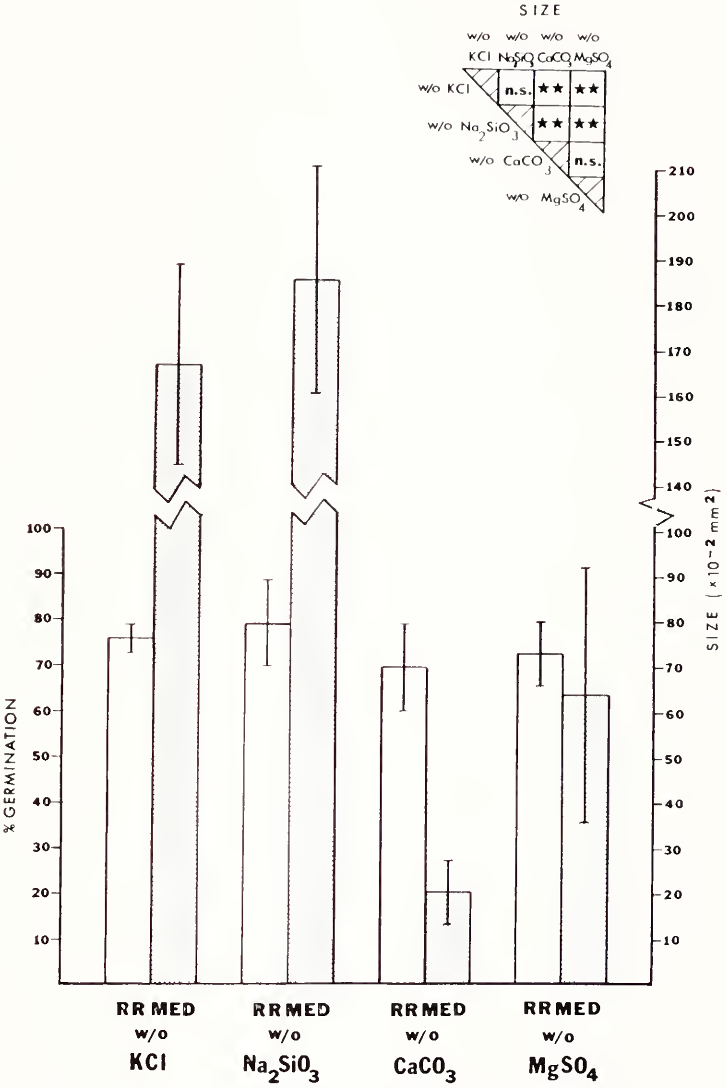

FIGURE 4. Response of *S. fragilis* gemmules to RR Med lacking each of its various components; conventions as in Figure 1.

DISCUSSION

Rasmont and Schmidt (1967) suggested that distilled water should provide a suitable medium for germination since they noted very little difference in respiration rates between gemmules of *Ephydatia fluviatilis* in it and artificial medium. It was hinted also that an increase in respiration relates to an increase in germination. Respiration was found to increase in light and decrease in dark for gemmules

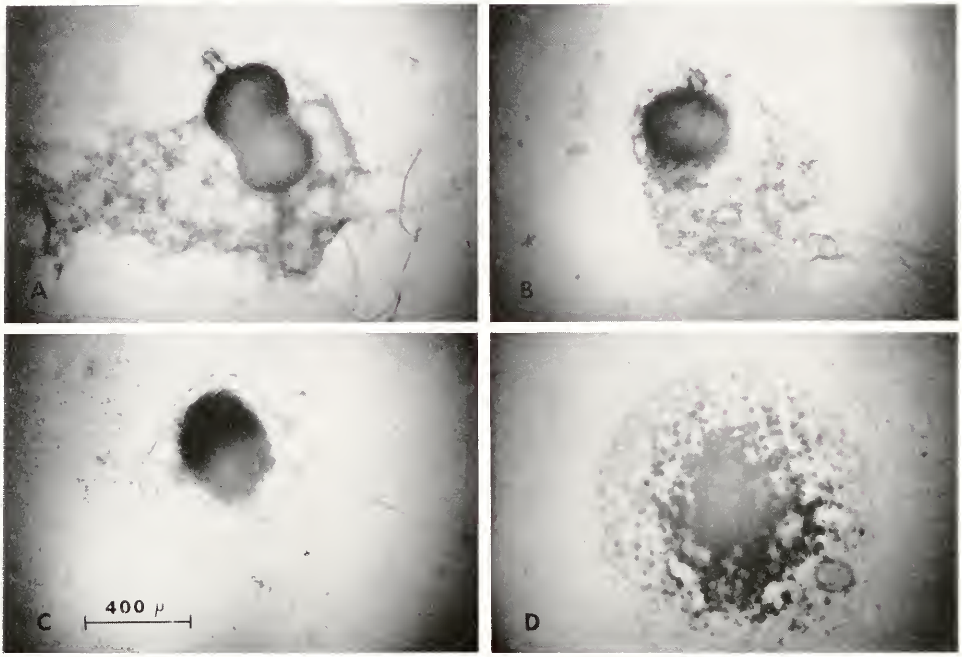

FIGURE 5. (A) Sponge germinated in RR Med lacking KCl; note spicules, osculum, and fine texture of cellular material; (B) sponge in RR Med lacking Na_2SiO_3; similar in morphology to A, but with conspicuous absence of spicules; (C) germinated gemmule in RR Med lacking $CaCO_3$; cells have barely emigrated beyond shell of gemmule; (D) cells from gemmule in RR Med lacking $MgSO_4$ have emigrated completely to form an amorphous, granular mass.

incubated in artificial medium. In addition, it was found that total light and total dark appear to be inhibitory to gemmule formation (Rasmont, 1970). The relationship of respiration to germination, however, may not be direct. Although green gemmules of *S. lacustris* respire the same in light and in dark, germination is somewhat slower in dark (Brønsted and Løvtrup, 1953).

Clearly, *S. fragilis* gemmules are not affected by light when grown in stream water or RR Med since germination and growth rates were similar in both light-dark and dark conditions. Penney (1954) stated that light does not affect the growth of freshwater sponges, but may prove limiting to symbiotic algae. Sponges in moving water tend to be located under submerged objects (Cheatum and Harris, 1953), and hence would receive very little radiation.

The effect of osmotic pressure on germination is uncertain. Rozenfeld's (1971) work on *E. fluviatilis* suggests that enzymatic action opens the micropyle and permits germination. Until that time the archaeocytes within the gemmule appear to be protected from osmotic rupture by the hydrostatic pressure of the shell. Gemmules artificially ruptured release a small percentage of archaeocytes, which may or may not differentiate depending upon the medium. It is assumed that rupture of the micropyle would also produce a small percentage of emigrant cells. If gemmules are impermeable, it is difficult to imagine why germination percentages from distilled water do not equal those from RR Med or RR water, since conditions of light, temperature, and duration of experiments were similar.

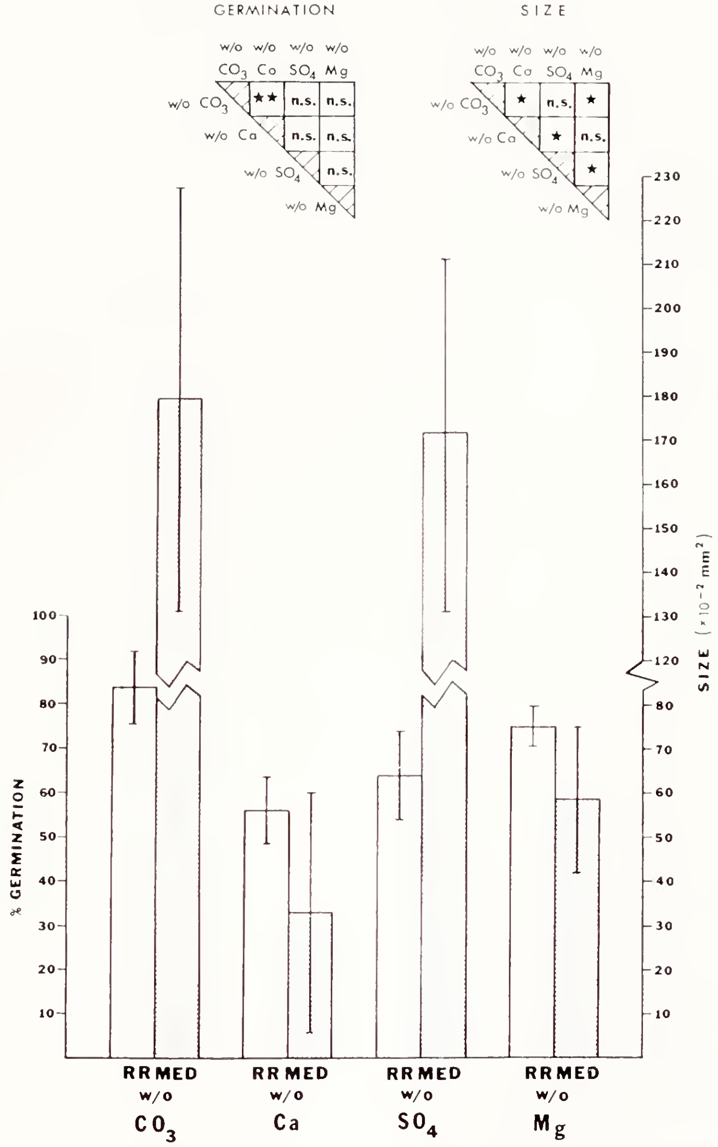

FIGURE 6. Response of *S. fragilis* gemmules to RR Med lacking individual ions; conventions as in Figure 1.

Freshwater sponges are believed to germinate between 13° and 15° C (Pennak, 1953). *S. fragilis* germinated in the laboratory at 6–8° C, and in the stream between 7.5° and 10.5° C; increasing temperature with the season would enhance the process.

Gemmulation, the formation of resistant gemmules which overwinter, began after a 5 month growing season at temperatures below 16° C; within 2 weeks thereafter the temperature had dropped back to 7–8° C. Rasmont (1968) summarized in part that the process depends upon genetic capacity, nutrition, and relative development in *E. fluviatilis*. It was impossible in this study to test the effect of temperature on gemmulation since the sponges were not fed.

Calcium and magnesium are important controlling factors for normal cell activity because they affect cell permeability (Ruttner, 1969). Although Humphreys (1963) found calcium to be totally satisfactory in facilitating normal aggregation of marine sponge cells, it is believed (Robertson, 1941) that both divalent cations are necessary for stabilization of intercellular matrices; distilled water has a disintegrative effect on cell aggregations. Van de Vyver (1971) noted, however, that the term intercellular matrix may not be applicable to an organism lacking specific tissues, and whose cells are migrating. Amoeboid movement occurs if either cation is present, but coalescence requires both. Galtsoff (1925) suggested that each divalent ion has characteristic functions which cannot be satisfied by the other.

Jewell (1939) concluded that calcium, and not magnesium, limits sponge distribution. Freshwater sponges in Wisconsin were found in water containing as low as 0.3 mg/l Ca and 0.0 mg/l Mg; however, *S. fragilis* was limited to 2.08 and 1.0 mg/l, respectively. Macan (1961) also included calcium (among others) as a limiting factor for freshwater organisms. Excess calcium also proves to be limiting (Robertson, 1941).

Jewell (1935) has noted that silica may be limiting in strongly skeletoned forms such as *S. fragilis*. Spicule production is poor below 1.0 mg/l SiO_2, but good growth can be obtained with only a trace (Pennak, 1953). *S. fragilis* has been found in water containing as little as 0.3 mg/l SiO_2 (Wurtz, 1950).

While spicules are the first structures to be differentiated after hatching (Rasmont, 1970), spicule formation is not necessarily a suitable criterion for judging development. Sponge morphology may be influenced by environmental factors other then SiO_2; for example, high organic content may produce rapid growth without spicule formation, and no spicule pattern may be considered normal since natural conditions may induce variation (Jewell, 1935; Racek, 1969). Much differentiation and growth was achieved in RR Med w/o Na_2SiO_3, but no spicules were formed.

Although calcium shows a tendency to support greater growth and differentiation than magnesium, both elements appear necessary for normal growth. RR Med/10 contains 0.2 mg/l Ca and 0.2 mg/l Mg (or 0.4 mg/l total ions) and results in a "normal" sponge; RR Med lacking either Ca or Mg (but still containing 2.0 mg/l total ions) produces a small granular mass.

Low concentrations of nutrients in a natural system may not be as limiting as expected since there would be constant replenishment due to current flow. In the preceding experiments a relatively short incubation period obviated replenishment.

The study was limited in time and scope. Since the sponges would have little or no opportunity to feed, extensive development was curtailed. Incubation was long enough to permit some growth, but short enough to prevent the cells from using up their energy reserves and disintegrating (Rasmont, 1961). Normal morphology suggests only that the organisms are functional.

The sudden appearance and disappearance of a species might suggest that one or a number of environmental factors are involved in limiting distribution. None of the important chemical parameters—calcium, alkalinity, silica, and pH (Pennak, 1953)—were limiting since there were no apparent differences among the three sample sites on any given date. The lake appeared to have negligible influence on water chemistry.

Since chemical factors remain consistent through the course of Rapid Run, it appears that physical factors limit distribution. Under constant laboratory conditions sponges tolerate lower concentrations of inorganic material than have been recorded in field surveys. RR Med is relatively dilute, and RR water is much cleaner than many systems found to contain this species. This in itself suggests the importance of physical factors, especially in a stream. The sponges found in the quiet water around the dam are protected from current, desiccation, and suspended material which are greater in the stream above and below the dam; these sponges probably seed the stream with gemmules which may proliferate under the favorable influences found below the dam for a short distance, but only on stable substrates. No sponges were found above the lake as such a small, swift stream would not prove inviting to aquatic birds, the probable transport agent.

SUMMARY

Gemmules of the freshwater sponge *Spongilla fragilis* were germinated and grown under a number of conditions in the laboratory using an artificial medium similar to stream water: germination was similiar under conditions of light-dark and total dark; germination was prompted by temperatures between 6–8° C; development of the young sponge was supported by ion concentrations as low as 0.2 mg/l for Mg and Ca, 0.05 mg/l for K and Na, and 0.06 mg/l for SiO_2; sponge development was inhibted by the absence of Ca and Mg, but not Na, K, or SiO_2, although no spicules were formed without SiO_2.

An annual survey was conducted to determine which physico-chemical parameters might limit the distribution of the species. As little difference with respect to chemical characteristics was noted among sampling stations and since sponges appear to tolerate low ion concentrations, it is suggested that distribution of sponges in the stream was affected by the interrelationship between current flow, suspended material, and substratum.

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STUDIES ON THE MAINTENANCE OF ADULT SQUID (*LOLIGO PEALEI*). I. FACTORIAL SURVEY[1]

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Attempts to maintain cephalopods under laboratory conditions are usually concerned with a need to stabilize the supply of animals for various biological studies. Related to these basic investigations is the hope of learning more about the stocks of cephalopods in order to better exploit them for human food or, possibly, domesticate them in some form of mariculture. At the present time, it appears that the maintenance of cephalopods is replete with problems unique to each taxonomic group, as the life history differences might suggest. Maintenance success to date is apparently directly related to the benthic association of the group; thus octopuses in general are easier to maintain than cuttlefish, which in turn are easier to maintain than true squid. From practical considerations, species chosen for maintenance purposes should be readily available, small (in adult size), rapidly attain sexual maturation, have simple food preferences, and be tolerant of aquarium conditions.

The present study reports attempts to evaluate factors relating to the maintenance of the common Atlantic Coast squid, *Loligo pealei* (Lesueur, 1821). This animal is seasonally available (inshore), relatively large, reaches sexual maturity in one year (Summers, 1971), has only partially defined food requirements, and is notably intolerant of aquarium conditions (Summers and McMahon, 1970). It does, however, possess large axons which are the standard material for a considerable body of neurophysiological research and, as of the winter of 1969–1970, it has become the object of a sizable offshore fishery. A factorial approach was chosen in order to provide quantitative information which might lead to evenutal improvements in long term maintenance of *L. pealei*. Though we were aware of several rearing studies under way or completed at the beginning of this work, and had useful communications with Drs. J. M. Arnold, S. von Boletzky and E. T. LaRoe, our principal sources of information were summarized along with results of preliminary studies in an earlier paper (Summers and McMahon, 1970). Under rudimentary conditions, our experience had been that the survival of *L. pealei* was approximately 71% per day (a half-life of two days) over a period of one week. We had not succeeded in demonstrating differentials in survival related to the method of capture, water temperature, size, sex, or level of crowding.

MATERIALS AND METHODS

Source

Live squid (*L. pealei*) were taken with otter trawl nets on day trips within 20 km of Woods Hole, Massachusetts. Fishing locations and details of some of the trawl

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equipment have been reported previously (Summers, 1968 and 1971; Summers and McMahon, 1970). Sixteen batches of squid were utilized. These resulted from captures ranging in date from May 11 to November 19, 1970. Ten batches were trawled by the R/V A. E. VERRILL, seven utilizing a 45–65 Long Island Sound balloon trawl (Summers and McMahon, 1970) and three utilizing a similar but smaller net designated as a 38–46 bar net by its maker, George W. Wilcox Company (Summers, McMahon and Ruppert, 1974). Six additional batches of squid resulted from trawling carried out by the commercial fishing vessel CAP'N BILL IV which utilized a #41 trawl net (see Summers, 1969). The first of these six batches was obtained by exclusive charter; the remainder were obtained through the Supply Department of the Marine Biological Laboratory which regularly employed the CAP'N BILL IV for summer squid collection.

Trawl contents were promptly emptied into seawater-filled tubs on the fishing vessels. Live squid were sorted into deck tanks (A. E. VERRILL) or a flooded fish hold (CAP'N BILL IV) and provided with running seawater until landed (a period of roughly one-half to four hours duration). Squid delivered by the Supply Department were subjected to repeated handling and sorting, often occupying as long as one hour, before being hand carried in tubs to the experimental tanks. Those obtained by direct charter of the CAP'N BILL IV and from the A. E. VERRILL were transferred into several 40-liter, polyethylene containers on the fishing vessel; these were filled to the top, fitted with tight lids (to prevent sloshing), provided with oxygen generating devices (O-Tabs), wheeled on hand carts, and finally hand carried to the experimental tanks. The entire procedure normally required about 20 minutes.

A total of 468 live squid were used in 16 experiments (341 males and 154 females). The disparate sex ratio resulted from sorting for large specimens. It was especially prominent among larger squid and among those obtained through the Supply Department. The numbers of squid used in the experiments varied from 16 to 48. Age composition estimated on the basis of population studies (Summers, 1971) suggested that 10% were young-of-the-year, 58% one year olds, and 32% two years olds. Young-of-the-year squid were predominant in the last four batches (capture dates: October 29 to November 19, 1970), one year olds were most common in the eight batches ranging from June 18 to October 15, 1970 and two year olds were numerous in the first 4 batches (May 11 to June 8, 1970).

Aquaria

Aquarium facilities for this study were built in a basement-level room approximately 6.7 m long by 6.1 m wide. Clerestory windows were provided on the northwest wall. These were fitted with venetian blinds and faced an adjacent brick building; thus, no direct sunlight shone into the room. The concrete walls and ceiling of the aquarium room were painted off-white and continuous, uniform illumination was provided by four tracks of fluorescent light fixtures located at ceiling level. Continuous air movement was maintained through the aquarium room and it was kept locked during experiments to avoid unnecessary disturbances. Convection heating/cooling units and nearby thermostatic controls held air temperature in the aquarium room to approximately 20–24° C.

Experimental aquaria consisted of four rectangular fiberglass tanks; two sizes each in two separate tank stands. A schematic presentation of the aquarium arrange-

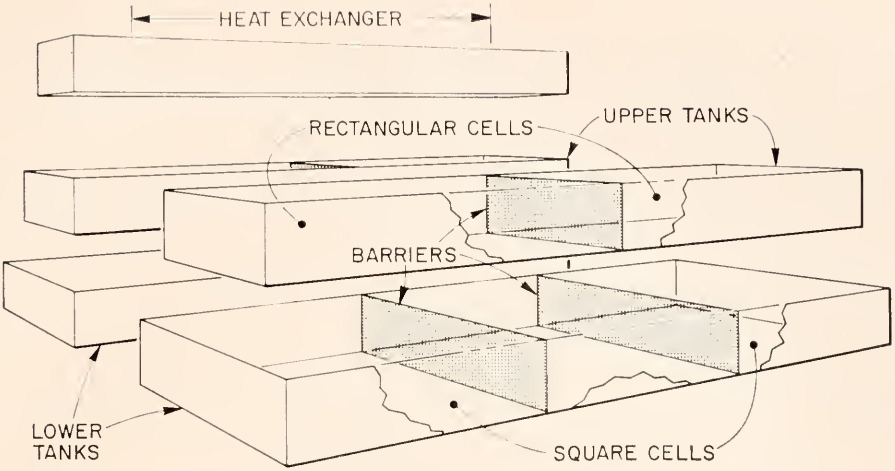

FIGURE 1. Schematic presentation of experimental aquaria in perspective as if viewed from the east. Windows in the aquarium room were located beyond the northwest ends of the tanks, at the right background. Portions of the northeast tank walls have been removed to show the position of barriers within the aquaria and the locations of square and rectangular experimental cells. Chilled seawater was available in the southwest tank stand (one upper and one lower tank) as a result of its passage through a reservoir containing a heat exchanger above these tanks.

ment is shown in Figure 1. All tanks had an inside length of 3.66 m. Lower tanks had an inside width of 1.37 m and upper tanks had an inside width of 0.92 m. Water depth was 0.31 m in all tanks, and the tank walls extended 0.05 m above water level. One tank stand was provided with an additional fiberglass reservoir of similar construction, 0.51 m wide, over the experimental tanks. All aquaria were opaque and coated with a smooth surfaced, buff-white gelcoat. They were made exceptionally rigid and partially insulated by the inclusion of $\frac{1}{2}$-inch (1.3 cm) end grain balsa core in the tank bottoms.

The reservoir held a galvanized steel heat exchanger consisting of ten vertical panels 2.72 m long by 0.30 m high. In conjunction with a $7\frac{1}{2}$ horsepower refrigeration compressor located outside the aquarium room, this heat exchanger functioned as an evaporator, allowing regulated cooling of the seawater entering one tank stand. The refrigeration unit could depress the temperature of a full flow of seawater about 10° C. Selected seawater temperatures were manually preset by adjusting the Freon pressure controls on the compressor. Sensitivity of the refrigeration unit was improved and (at low temperatures) formation of ice on the heat exchanger eliminated by reducing stratification of seawater in the reservoir. This was accomplished by running a stirring motor near the downsteam end of the heat exchanger and perforating the reservoir standpipe along its length with a number of small holes. Seawater temperatures in the chilled tank stand generally remained within a fractional degree Centigrade of preset temperatures.

Seawater was provided at one end of the upper tank and reservoir through polyvinylchloride (PVC) supply lines from the Marine Biological Laboratory seawater system; seawater passed the full length of each tank before cascading to the next lower level via "2 inch" PVC standpipes. Flow was held at approximately 2000 liters per hour in each tank stand. Higher flow rates produced considerable disturbance in the vicinity of the standpipes. This flow provided roughly two complete changes per hour in the upper (0.92 m wide) tanks and one and one-third changes per hour in the lower (1.37 m wide) tanks. Aeration occurred each time the seawater cascaded from one level to the next; mixing was aided by bubbling at the inlets and by continuous activity of the squid. Because the aquaria were new fabrication, they were cured at room temperature in air for several months, soaked in rapidly flowing seawater, and scrubbed periodically for a few weeks to dissipate any remaining solvents before the initiation of experiments.

All experimental tanks were fitted with lids made from corrugated fiberglass roofing material laid across the width of the tanks, overlapping where panels met and extending beyond the top tank edges. This material was selected for a spectral transparency similar to tabulated values (Svedrup, Johnson and Fleming, 1947) of clear coastal seawater. (In fact, except for a higher transparency in the red end of the visible spectrum, it closely mimicked typical values listed for one meter of coastal seawater.) The fiberglass material had the advantage of diffusing light and reducing sharp silhouettes. Lids mechanically prevented the escape of squid and very likely reduced the exchange of water with room air. Their presence also reduced possible tank fouling by airborne dust.

Movable barriers were constructed to divide the tanks into smaller compartments. These were made from rectangular frames of unpainted softwood lumber with knotless Nylon netting material (1.3 cm stretched measure) stapled over one side. Barriers were wedged inside the tanks with narrow splits of cedar shingles. In conjunction with the tank lids, the barriers effectively restricted squid to preselected segments of the experimental tanks.

Design

The experimental design called for the identification of factors significant in affecting the longevity of adult squid maintained under laboratory conditions. Because the supply of live squid was unpredictable, and earlier experience indicated that maintenance experiments might well extend beyond one week (Summers and McMahon, 1970), the design had to be both efficient and flexible. The basic design chosen was a 2^3 factorial experiment in which three factors were studied simultaneously, each at two different levels. Replications were to be provided within experiments in amounts depending upon the availability of live squid. Furthermore, experiments were to be repeated in suites of similar design in order to extract information on differences between batches of squid and maximize the evaluation of various factor combinations. Analysis was to be accomplished through an analysis of variance (ANOVA) of each individual experiment and of the pooled data from suites of experiments. Due to the seasonal nature of squid (Summers, 1969 and 1971) and ambient conditions in their native habitat (*e.g.*, seawater temperatures), it was anticipated that a number of observations could be made during the course of the experiments on factors which were independent of any particular experimental design. To this end, special care was taken to obtain the additional pertinent data.

It was recognized in advance that the two levels of each factor would have to be distinctive in order to properly evaluate the factor and that interactions beyond the first order would be poorly tested. Additionally, the 2^3 factorial design required a minimum of eight experimental "cells" necessitating the use of barriers, as described above and shown in Figure 1. Both upper tanks were divided in halves by single barriers at the midpoint, creating four "rectangular cells," 0.92 × 1.83 m by 0.31 m deep. Both lower tanks were divided in thirds with two barriers each, forming six "square cells," 1.37 × 1.22 m by 0.31 m deep. (Though not precisely "square," these cells were a reasonable approximation and crudely represented a regular polygon in contrast to the rectangular cells.) All cells had a plan area of approximately 1.68 m^2 and a capacity of approximately 520 liters. Because the four rectangular cells had only one netting wall each (the barrier), it was decided to use the four comparable square cells in the lower tanks for the experiments. Thus, the two center cells in the lower tanks (with two netting walls each) were not commited in the 2^3 factorial experimental design, and they were often used for the storage of spare squid. One odd experiment (the eleventh in consecutive order) was run in conjunction with the end of the previous experiment; it utilized the six square cells in a 2 × 3 factorial design.

It follows that any 2^3 factorial experiment utilizing these eight cells has cell shape as one factor, contrasting rectangular *vs.* square configurations at its two levels. When the refrigeration equipment was operated, another necessary factor was relative water temperature; contrasting ambient *vs.* a preset (chilled) water temperature. In operation, the chilled temperature was adjusted every few days to hold approximately a 5° differential between the two levels of this factor. The third imposed factor in the fifteen 2^3 factorial experiments was either feeding (levels: unfed *vs.* fed two live fish, *Fundulus* spp., per squid per day) or crowding (numbers ranging from one to eight squid per cell at paired levels determined by the available supply). The upper level of the feeding factor was arbitrary, though based on previous observations and was modified after the sixth experiment to prevent an indefinite accumulation of fish. The new level responded to demand by replenishing *Fundulus* daily, but not exceeding two per squid. Six experiments had the factors cell shape, feeding and crowding; six had the factors cell shape, feeding, and relative water temperature, and three had the factors cell shape, crowding, and relative water temperature. Where feeding was not a factor, all squid were fed as in the modified upper level given above. The 2 × 3 factorial experiment crossed relative water temperature with three square cells which were arbitrarily designated by their order relative to seawater flow.

During experiments, observations were made on an average of once every 9½ hours and no less than two hours nor more than one day separated consecutive observations. Typically, three observations were made during the working day (about four hours apart) and three over the weekend. Sporadic observations were made at other times during the course of these experiments. Elapsed time (to the nearest tenth of an hour) from the start of the experiment, date and time of day, barometric pressure, air temperature, ambient seawater temperature and chilled seawater temperature were logged at each observation; the last two were measured in the experimental tanks. Necessary operations were performed in or near the tanks and an accounting was taken of all squid at each observation, with as little disturbance as possible. Dead squid were removed, their dorsal mantle length, sex and sexual maturity were recorded with any comments on their condition. Survival time was considered to be the total elapsed time from being placed into the experimental tank to the observation of death; this was the principal statistic for analysis.

Operationally, tanks were drained, scrubbed (with bristle brushes and Nylon scouring pads but without chemicals), hosed down with fresh water and refilled just before another experiment began. Superfluous organic material of all forms, fresh squid eggs excepted, was removed at each observation. The experimental design was set and treatment combinations were randomized among cells before the experiments. (Because of physical restrictions, relative water temperature could not be crossed with tank stands and cell shape could not be crossed with upper and lower tanks; these factors were in fact nested.) On delivery, squid were immediately and impartially distributed among the experimental cells to the maximum number of whole replicates that the supply allowed. Refrigeration equipment was not started until the lids were in place and the squid had had a few minutes to adjust to the tanks. Chilling of the one tank stand produced measurable but transient temperature stratification which progressed first in the upper, than in the lower tank. It normally required 2–3 hours before stabilizing at the preset temperature. Feeding was begun on the morning following initiation of the experiment and was repeated daily at the first observation. Electrical services for the aquarium room, including the lighting, ventilating fan, and refrigeration system, were on circuitry which received emergency power in case of outages; these appliances were left on continuously and apparently functioned with no more than momentary failures through the 148 days required for the 16 experiments.

RESULTS

Mean survival of all squid was 87 hours (approximately 3$\frac{2}{3}$ days) and individual experiments had mean survival times ranging from 58 to 142 hours. The maximum survival of an individual squid was 666 hours. There was mild statistical evidence for greater variability in survival times between batches of squid than within experiments. Analyses of variance demonstrated better than 95% confidence of significance for the factors cell shape, feeding and relative water temperature and the first order interaction of feeding and relative water temperature in at least one experiment each. Cell shape and relative water temperature produced better than 99% confidence of signficance in different experiments. Cell shape was the only factor demonstrated to have better than 95% confidence of significance in pooled data resulting from suites of similar experiments (it actually produced better than 99% confidence of significance).

A review of the data showed that rectangular cells promoted longer squid survival than square cells with a mean difference of nine hours overall. By construction, cell shapes were nested within tank heights, and these findings are confounded with the upper and lower tanks. No significance was demonstrated through the application of a chi-square test on pooled survival data for upstream *vs.* downstream pairs of cells in the same tank nor among cumulative data for the eight specific cells. The 2 × 3 factorial experiment did not show significance for the different square cells.

Seawater temperature ranged from 5.8–6.2° C to 20.1–22.6° C through the course of these experiments. Where relative water temperature was found significant (three out of a possible ten experiments), levels favoring survival differed.

The 2 × 3 factorial experiment indicated better than 99% confidence of a significance for relative water temperature, strongly favoring survival at the chilled level (11.1–11.7° C) contrasted with the ambient level (18.0–18.4° C) in a case where temperature differential was deliberately exaggerated. Attempts to correlate survival with absolute water temperatures were inconclusive. However, it was noted in the observations that squid kept at temperatures below 8° C behaved aberrantly and did not appear to feed readily (see Summers, 1969).

The crowding factor was studied at lower levels of two, three, and four squid per cell and higher levels twice those numbers in two experiments each (a total of six experiments). In addition, isolated individuals were contrasted with three, four and five squid per cell in one experiment each. In none of these nine cases was crowding demonstrated as a significant factor.

Feeding was utilized as a factor in twleve experiments and was found significant in only one. This, and the isolated occurrence of significance in one case of a first order interaction, can be attributed to chance.

There was mild statistical evidence (better than 90% confidence) for a difference between fishing vessels and their gear in the ultimate survival of squid. On an average, those trawled by the CAP'N BILL IV (and handled by the Laboratory Supply Department) survived ten hours less than ones trawled by the A. E. VERRILL.

Discussion

As can be expected, some aspects of the experiments had to be accommodated through modification of the experimental procedure. For example, the refrigeration unit was not completely installed and operational until the seventh experiment, which began on August 5, 1970. However, this corresponded with the occurrence of maximum ambient seawater temperatures and allowed study of the relative water temperature factor over the full range of ambient temperatures. The potential for ice formation in the reservoir was learned by experience and the stirring motor was not used until early November (during the fourteenth experiment). Though temperature control was maintained, it was found desirable to reduce the seawater flow rates during some coastal storms in order to minimize turbidity in the tanks.

During the first two experiments, four squid succeeded in jetting out of the tanks in spite of the lids. Because these "escapes" occurred in the first 2 days of both experiments, the squid were replaced by spare animals from the same batch which had been held at appropriate seawater temperatures in the lower tanks and were, at that stage, unfed. Various pieces of lumber were placed on top of the lids at this point, adding weight and reducing flexibility of the fiberglass materials; this prevented further squid escapes. Excepting the four escaped squid, all causes of mortality were accepted as appropriate experimental results. This included a few squid which were sufficiently weakened to be swept down the standpipes and were recovered on grating in the drain system.

Dead squid were frequently found in the corners of the cells and typically had damaged tail ends. This condition was manifest by abrasion of the skin and/or open wounds usually exposing the fractured tip of the pen. These animals showed progressive blunting of the tail end during maintenance, accompanied by an upward twisting at the tip. This condition is apparent in photographs of squid published by Voss and Sisson (1967). Some dead squid had been cannibalized, causing difficulty in determining their exact size and sometimes their sexual development. It was observed that moribund individuals were occasionally set upon by others, and this behavior was not correlated with the level of feeding. As reported previously (Summers and McMahon, 1970), cannibalism represented a limited, though real, source of nourishment for unfed squid.

Because computational facilities were available for the analysis of variance of 2^2 experimental designs having equal numbers of replicates in each data cell, the experimental results were regularly run three times, once for each pair of factors. Where crowding was one of the factors, some data was necessarily eliminated (by a random process) and the statistical results are conservative. The most powerful analysis of a factor pair was utilized in determining significance in any single body of data.

The presence of a galvanized heat exchanger in the seawater flowing to one tank stand was viewed as a potential factor in the survival of squid. Effects of zinc leaching from the heat exchanger were experimentally confounded with relative water temperature, which was not found to be frequently or consistently significant. In addition, the heat exchanger was not present in the first 6 experiments and a one-way analysis of variance for survival in the one tank stand, before and after its installation, was not significant. An analysis of dissolved zinc was performed by Dr. Derek Spencer of the Woods Hole Oceanographic Institution; it showed a ten-fold increase in the seawater flowing through the chilled tank stand compared with ambient seawater (44.0 ± 3.5 micrograms of zinc per liter compared with 4.5 ± 0.4 micrograms of zinc per liter). Reference was made to several published analyses of seawater, all of which gave zinc concentrations for typical coastal seawater intermediate to these two figures.

Failure to determine significance in the crowding factor was disappointing. Though it was observed that healthy undisturbed animals swam synchronously in groupings relatively smaller than the experimental cells, it had been anticipated that crowding would influence mechanical interaction with the tank walls and/or alter behavior patterns, thus altering survival. None was experimentally detectable. The isolation of individual squid in three experiments should have provided the strongest test for this factor, yet no effect was found. Nor was it possible to correlate the survival of squid with initial crowding using the pooled data from all the experiments. The isolation of one sex in a particular experimental cell by chance (23 cases) and by design (12 cases; isolated squid) did not produce survivals which were testably different from the experimental means, nor were survival differences between isolated males and isolated females found significant through the use of chi-square tests.

In our earlier work (Summers and McMahon, 1970), feeding had been avoided in order to study short-range effects. In the present study, feeding did not measurably improve survival. When fed, squid attacked and consumed live *Fundulus* in a deliberate fashion and defecated as evidence of digestion. It is possible that our imposed level of feeding was actually no better than starvation, thus preventing a statistical test of significance. We found it necessary to put *Fundulus* in the experimental cells one (or a few) at a time and observed that healthy squid would immediately seize them and withdraw in what appeared to be the peck order of that particular group. Proximity could alter this order of feeding, sometimes resulting in a dispute between squid. When several *Fundulus* were added simultaneously and/or were not taken when first presented, a single squid sometimes seized a number of them. In these cases, we were likely to overfeed in order to provide for the remaining squid. When moribund, squid often would not feed, hence our modification of the feeding level to prevent the accumulation of fish. The longest lived squid were in treatment combinations which included feeding. In view of these sparse facts, we hesitate to suggest that squid can be expected to survive indefinitely without feeding, but note that our results are anomalous.

Analyses of survival by age groups became simultaneously a study of survival by size because age groups were identified by size. Furthermore, different age groups appeared at different seasons, and one group (one year olds) had a seasonal change in sexual maturity (see Summers, 1971). For these reasons, Figure 2 shows survival by age group, size, sex, season and the significant factor, cell shape. As seen in part A of Figure 2, sexual dimorphism in dorsal mantle length becomes greater with age, as does the apparent effect of cell shape in the survival of squid. On overall mean, neither sex has a distinct survival advantage for any one age group. Insets in part B represent the range of survival times (shown in part A) and the period of principal occurrences for young-of-the-year and two year olds. Data for one year olds has been separated into two month periods in part B. Where seasonally mixed, it appears that older (larger) age groups have a survival advantage; especially late in the year when one year olds and young-of-the-year squid occur together. One year old males appear to survive longer than contemporary females during the first half of the year when they are both sexually mature and breeding.

We do not fully grasp the consistent significance of cell shape in the survival of squid. Were it not for the casual observation that squid oriented randomly relative to major cell dimensions, we might suggest that tank length determined this effect. Though differing in maximum length by nearly one-half meter, cell shapes were within one-quarter meter of the same diagonal measure and otherwise nearly equivalent. Order of a cell in the flow pattern did not appear to affect survival nor did the number of squid upstream from the experimental cell.

In a related study early in the season, a number of large squid (sexually mature males, 30–35 cm in dorsal mantle length) were driven the length of a long fiberglass tank to determine their swimming speed. the tank was 15.24 m long by 0. 92 m wide, filled to a depth of approximately 0.50 m and the timing course was the centrally located 12.19 m portion. When frightened by a brightly colored paddle, squid could be driven down the tank. Squid were usually clocked two or three times each before they tired (becoming slower in successive runs). Some evenutally attacked the paddle instead of completing the course. When fresh, these large squid swam at a sustained rate of approximately one meter per second, moving a mean one and one-third meters per jet pulse. This is consistent with calculated initial velocities of squid jumping free of the water, which were estimated at about 2 m/sec (*L. pealei,* Cole and Gilbert, 1970; *Loligo vulgaris,* Packard, 1969). Similar tests with smaller squid, including several females, showed that speed was roughly proportional to size, though jet pulse rate was the same for all sizes. If the maximum locomoter jet pulse of a squid is an "all or none" event (which the structure of its mantle nervous system suggests), then the "squid mean free path" appears to be no more than $1\frac{1}{3}$ m and likely about $\frac{2}{3}$ m for a typically-sized squid.

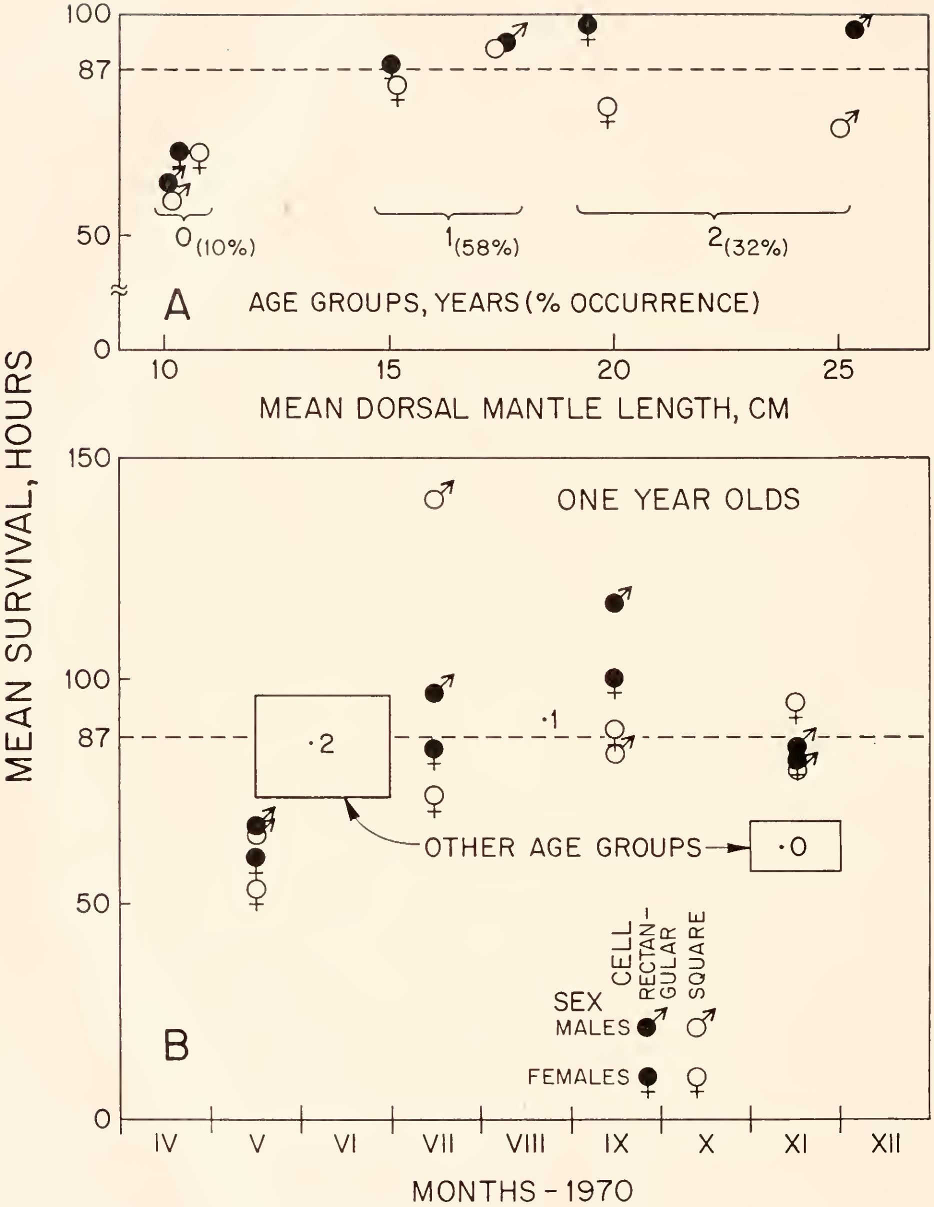

Figure 2. Mean survival of squid by size, age, sex, experimental cell shape and season. The overall mean, 87 hours, is indicated by a dashed line in both parts of the figure. Insets in part B represent the seasonal occurrence of a particular age group in the experiments and the range of survival times shown in part A, above. Data for the one year olds has been broken down into two month periods in part B. See text for discussion of the figure.

These figures are close to the dimensions of the experimental cells and may aid in evaluation of both absolute size and shape of maintenance tanks. (Note, a theoretical analysis of jet propulsion with reference to the squid *Loligo vulgaris* has been provided by Johnson, Soden and Trueman, 1972.)

Using the size data shown in Figure 2A, young-of-the-year squid, one year olds and two year olds had mean sizes (dorsal mantle lengths) of 10.5, 17.0 and 23.5 cm, respectively. Based on the foregoing empircal data, their respective sustained swimming velocities should be approximately 0.35, 0.57 and 0.78 m/sec. And, utilizing an approximate weight/length relationship (weight doubling for every 5 cm increase in dorsal mantle length; Summers, 1969), one can calculate kinetic energies of the different age groups at 15%, 100% and 470% relative to that of one year olds. With likely "squid mean free paths" of roughly 0.50, 0.75 and 1.00 m, respectively, the potential for collision damage obviously increases rapidly with size (and age). Given a hypothetical initial location central in any experimental cell and a random horizontal orientation, the chance that a young-of-the-year squid will strike the tank wall in one pulse is zero while that for a two year old is 100%. One year olds have probabilities of striking the tank wall of 31% in square cells and 53% in rectangular cells under these conditions. (Note, two-dimensional derivations of the ideal gas law also suggest more frequent collisions in oblong cells.)

Reference to Figure 2 shows that cell shape was especially important in survival of older age groups (larger squid), as expected, but favored rectangular cells in which collisions should have been more frequent. Netting walls in the barriers were 1½ times longer in the square cells, though their presence was not judged detrimental as tested by the 2 × 3 factorial experiment. Thus, we are bound to suggest that squid may orient in aquaria in such a way that they minimize collisions with the walls. Collison models built on these empirical data assume a horizontal plane of movement and provide logical mechanisms for the proportional survival model suggested in our earlier paper (Summers and McMahon, 1970). Present survival data, however, is not as well fit by the previous exponential model and the mean survival is longer.

Additional information on this subject and acknowledgments are given in Summers, McMahon and Ruppert (1974).

SUMMARY

1. This paper reports a factorial survey of conditions affecting the survival of the squid, *Loligo pealei,* in laboratory aquaria. Running seawater and continuous illumination were provided to various numbers of animals kept in 520 liter experimental "cells." A total of 468 squid were studied in 16 separate experiments run between May 11 and November 23, 1970.

2. Three imposed factors were studied simultaneously in any one experiment. These included feeding, crowding, relative water temperature and "cell" shape. The effects of uncontrolled factors such as ambient seawater temperature, age composition, size and sexual maturity were also evaluated.

3. Mean survival of all squid was 87 hours and maximum survival was 666 hours. Analyses of variance demonstrated better than 99% confidence of significance in the factor, "cell" shape (rectangular "cells" produced longer survival

than square ones). Mixtures of dissimilar age groups appeared to be detrimental in the survival of the younger group, and breeding animals (one- and two-year olds) did not live as long as some sexually immature individuals.

4. Collisions with the aquarium walls are discussed as likely causes of mortalities. Behavioral observations are used in evaluating the probabilities of these collisions.

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STUDIES ON THE MAINTENANCE OF ADULT SQUID (*LOLIGO PEALI*). II. EMPIRICAL EXTENSIONS[1]

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GREGORY N. P. A. RUPPERT[4]

This study is a direct continuation of previous work on the maintenance of adult *Loligo pealei* (Summers and McMahon, 1974) which, in turn, benefited from a preliminary study (Summers and McMahon, 1970). The present study represents a second operational year of the facilities described in part I (Summers and McMahon, 1974); reference should be made to that paper for descriptions of the equipment and many experimental conditions. Reports on related studies appeared subsequent to planning for the present work (von Boletzky, von Boletzky, Frosch and Gatzi, 1971; LaRoe, 1971; Arnold, Singley and Williams-Arnold, 1972; Mikulich and Kozak, 1971). These were mostly concerned with squid rearing, though LaRoe (1971) reported on the rearing and maintenance of the loliginid squid, *Dorytheuthis* (= *Loligo*) *plei* and *Sepioteuthis sepioidea*. Appropriately, other studies utilized species more tractable to laboratory maintenance i.e., Sepiolinae (5 species, von Boletzky, *et al.*, 1971) and the sepiolid, *Euprymna scolopes* (Arnold, *et al.*, 1972) Mikulich and Kozak (1971) reported briefly on the maintenance of an oceanic squid, *Todarodes pacificus*.

Materials and Methods

Aquarium modifications

Previous experience indicated that squid were injured through collision with the aquarium walls and suggested that this might contribute to early mortality (Summers and McMahon, 1970 and 1974). A number of tank modifications were suggested by investigators at the Marine Biological Laboratory with the intent of minimizing damage resulting from these collisions; none of them had been critically tested and some were completely untried. In our observations, vertical tank walls were most in need of modification and we thought survival improvements might be made if the walls could be made both smooth and yielding. We had little information on optical properties of the wall which might reduce the numbers of collisions.

A bumper system was developed through trial construction which provided several desirable features; this is shown in Figure 1. It consisted of clear polyethylene tubing, 3½ mils (0.90 mm) in thickness and 18 inches (46 cm) in flattened diameter hung vertically approximately 3 cm inside of the tank walls. The lower edge was held down by threading in two strands of lead-core line, the type used as a lead line for small seine nets. The top edge was alternatively taped to the flanged

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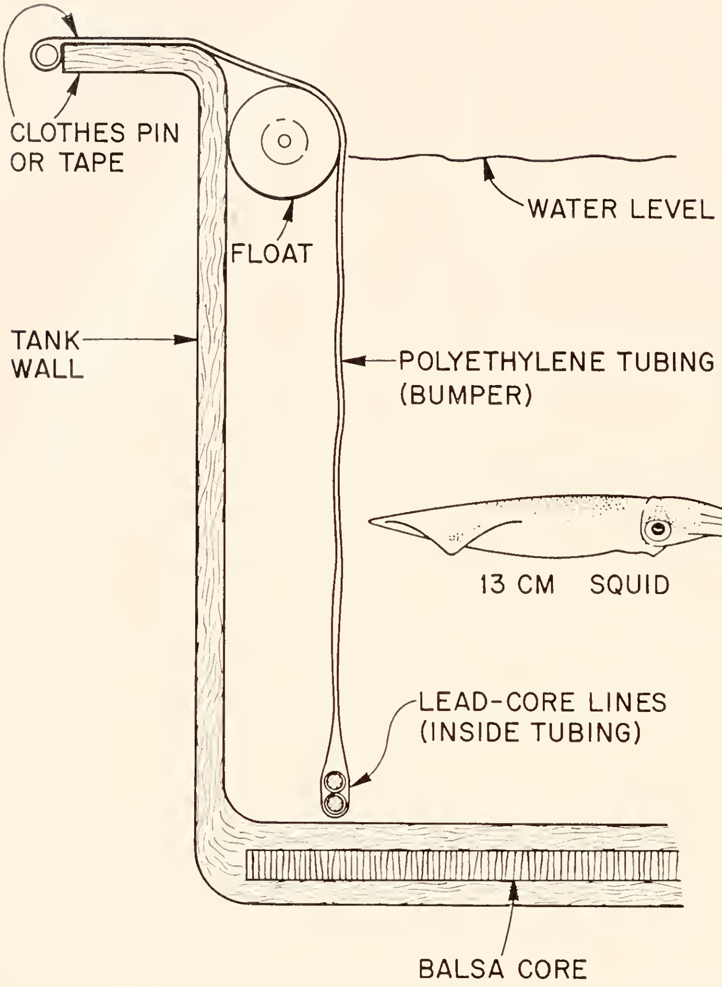

FIGURE 1. Typical cross-section of the bumper system at a tank wall. Squid colliding with the bumper must express seawater from the space between the polyethylene tubing the tank wall before striking a solid obstacle; this provides viscous damping of their kinetic energy. The bumper returns to its original position after a collision.

tank edge or clipped to it by threading a small polyethylene line through the tubing and attaching clothespins at close intervals. Adjustments were made at the tank flange to obtain a uniform, straight hanging surface all around the tank, and the ends of the tubing were overlapped with the inside lap folded back on itself. Spacing relative to the tank walls was accomplished by 13 × 3 cm hard plastic seine floats strung end to end on a small Nylon line at the water level between the wall and the polyethylene tubing. During the last three experiments, the bumper system was modified by eliminating tank corners. In this case, the upper edge of the polyethylene tubing was run at a 45° angle across each corner, thus excluding (in plan view) four isosceles triangles 30 cm on a side from the tank. The float line was shortened in this instance to take up slack tubing, effectively further rounding the corner.

The bumper system afforded tremendous viscous damping to any direct blow caused by squid and quickly returned to its original configuration after an impact. Water trapped behind the polyethylene tubing had to be forced out of a narrow space before the bumper would collapse; the more rapidly this was attempted, the more damping provided. Though hard, the floats were both light in weight and ellipsoidal, presenting little obstacle to a collision. The polyethylene tubing was visible in seawater and quickly acquired a noticeable microflora. When used simultaneously, bumpers were necessarily collapsed in the immediate vicinity of barriers.

Sources

Live squid were taken in the vicinity of Woods Hole, Massachusetts on eight separate occasions ranging in date from May 18 to November 10, 1971 (see Summers and McMahon, 1974). All but the third consecutive batch were trawled by the R/V CIONA, utilizing a 38–46 bar net (George W. Wilcox Company, Mystic, Connecticut). This otter trawl net was made from 15 thread Nylon netting material with a mesh size (stretched measure) of 10 cm. The cod end was lined with a knotted, soft-lay cotten netting material having a mesh size (stretched measure) of 3.5 cm. The trawl was fished from a single warp with 15 m legs, wooden doors (weighing 50 kg each) and a towing bridle 37 m long on each side of the towing swivel. A steel hoop between the cod end and liner prevented crushing of the catch, and the CIONA was especially rigged for quick emptying of the net into seawater-filled sorting tubs on the deck. Handling aboard ship and from dockside to the experimental tanks has been previously reported (Summers and McMahon, 1974).

The third batch of squid was taken in Great Harbor, Woods Hole, Massachusetts, adjacent to the National Marine Fisheries Service dock on the night of June 11–12, 1971. Lighting was rigged on the dock to attract prey animals; when the squid appeared, they were encircled with a 61 m (long) by 3.5 m (deep) seine net constructed of knotless Nylon netting with mesh sizes of 5 cm (stretched measure). Squid were bailed into a netting pound and transferred to the experimental tanks (the next morning) by the same method used for trawl-caught squid.

A total of 246 live squid were used in eight experiments (126 males, 106 females and 14 with no sex data). Age composition for the sexed squid was estimated from population studies (Summers, 1971) to be 10% young-of-the-year, 62% one year olds and 28% two year olds. One and two year olds were mixed in the four batches ranging in capture dates from May 18 to June 29, 1971. One year olds were mixed with young-of-the-year in the two batches caught on September 22 and November 19, 1971. Unmixed one year olds were taken in the two batches of August 4 and August 18, 1971.

Design

The experimental design called for an approach to the maximum possible squid survival through a series of internally sound experiments. Flexibility between individual experiments was to be exploited in order to attempt new innovations. At least one series of experiments was to be capable of determing the variability in survival within and between batches of squid. In contrast to the previous year's work (Summers and McMahon, 1974), a few obvious, though unproven, factors would be accepted as logical requirements. Thus, all squid were to be fed (on demand, to the level of two live *Fundulus* spp. per day) and crowding would not be used as a factor. Insofar as the supply of squid allowed, crowding was to be constant from experiment to experiment at numbers which assured good statistical evaluation. Relative water temperature was to be carefully studied, as was the shape and size of the maintenance tanks. The basic 2^n factorial experimental design was to be employed, examining several (n) factors at two levels each. Replicates were to be provided, and analysis was to be conducted by an analysis of variance (ANOVA) of individual experiments and of pooled data from suites of similar experiments. Pertinent data would be kept to provide a basis for the analysis of several conditions not employed as experimental factors.

Due to mechanical problems, seawater temperature control was not available during the first 2 experiments and the relative water temperature factors was not tested. Instead, a 2^2 factorial experimental design was employed which tested the factors bumpers and dividers (levels in both cases were with *vs.* without the particular devices). Because two basic tank shapes were used, this design provided tests of three experimental cell volumes (520, 1040 and 1560 liters) and five cell shapes (0.92 × 1.83, 0.92 × 3.66, 1.37 × 1.22, 1.37 × 2.44 and 1.37 × 3.66 m, all with water depths of 0.31 m). Crowding was held constant at three squid per 520 liter unit volume.

The third experiment had a 2^3 design; it was run as an incomplete block with the highest order interaction confounded with the two blocks. In other words, one-half of the experiment was run by sacrificing a test for the highest order interaction. Four tanks were used without dividers and the three factors were bumpers (levels: with *vs.* without), relative water temperature (ambient *vs.* chilled approximately 5° C below ambient) and tank levels (upper *vs.* lower). (With undivded tanks, the term "experimental cell" or simply "cell" is synonymous with "tank.") Squid for this one experiment had been caught in a seine and crowding was held at four squid per 520 liter unit volume.

The fourth through eighth experiments had a 2^2 design with factors tank levels and relative water temperature. Bumpers were used in all tanks and bumper corners were rounded in the last three experiments, as dscribed above. Crowding was maintained at three squid per 520 liter unit volume (30 squid in all) except in the fifth consecutive experiment, when fishing conditions had been poor and one less squid was used in each tank (for a total of 26 squid).

As in work of the previous year (Summers and McMahon, 1974), observations were made on an average of once every 9½ hours, and data logging was unchanged. Operational aspects of the experiments (*e.g.*, tank cleaning, physical conditions and randomization between experiments) was as previously reported. The principal statistic was the elapsed time at which an individual squid was found dead.

With minor exceptions, the facilities functioned without failure for the 179 days required to complete the eight experiments. Seawater flow stopped briefly once (September 28) with no apparent effect on the 16 squid then occupying the seventh experiment. In two experiments (the fourth and seventh), individual squid introduced a new problem by their prolonged survival. Because it was inefficient to commit the facilities indefinitely to single squid, these individuals were moved to another tank 16 and 8 days, respectively, after the death of the next longest lived batch-mates (surviving an additional 12 and 17 days, respectively). A standard Marine Biological Laboratory (fiberglass) sea table (of approximately 150-liter capacity) was the substitute tank and, excepting continuous illumination, no special attempt was made to duplicate experimental treatment combinations.

RESULTS

Mean survival of all squid was 177 hours (approximately 7⅓ days) and individual experiments had mean survival times ranging from 90 hours to 371 hours. Maximum survival of individual squid was 1124 and 1400 hours, for one year old, sexually mature female and male squid, respectively. No statistically significant results were detected in the first or second experiments testing bumpers and dividers (nor in pooled data from the two experiments), although there was an apparent direct correlation between survival and the absolute size of the experimental cell. In the third experiment, the survival advantage of the bumper system was demonstrated with better than 99% confidence of significance. In the last five experiments, tank level and the first order interaction of tank level and relative water temperature were found significant with better than 95% confidence in one experiment each. No significance was detected in the pooled data from this suite of five experiments, nor was there a signficant difference in the survival between *vs.* within batches. Although not statistically significant (except in one experiment), upper tanks regularly produced longer survival than lower tanks.

Minimum seawater temperatures were not artificially depressed below 8° C because earlier experience indicated that these were unnaturally cold for *L. pealei* (Summers, 1969; Summers and McMahon, 1974). Extreme range of seawater temperatures in the experiments was 8.6–10.0° C to 20.9–23.0° C. Relative water temperature was not found significant in any experiment or suite of experiments, nor was there a correlation found between absolute temperature and survival.

DISCUSSION

No experiment during 1971 had a mean survival time as low as the mean of the previous year's results (87 hours). The probability of this occurrence in the same population is one in 256, suggesting that a distinct change was brought about in 1971. An apparent evolution in experimental design may be seen in the results. The first two experiments were run without temperature control and failed to show the value of the bumpers, but gave a mild indication of a correlation between absolute cell size and survival (a least-square fit of the data gives a 7% increase in survival time for each 520-liter unit volume increase in experimental cell). Thus, the third experiment with factors: bumpers, (undivided) tank levels and relative water temperature. Determination of high significance for the bumper system coupled with an estimated 90% significance for the bumper/divider interaction in the second experiment, suggested that the value of bumpers was less discernible in small cells. The final experimental design, then, incorporated bumpers while continuing to test relative water temperature and tank levels. Though no consistent significance was found among these factors in the last five experiments, a two-fold improvement in survival time was realized.

The mean survival times of the fourth through eighth experiments were: 205, 100, 371, 221 and 195 hours, respectively. As stated earlier, the fourth and seventh experiments benefited from one especially long-lived squid each (whose ultimate, although modified, survivals were 5–6 times longer than their respective batch means). The distribution of survival times for the sixth experiment was more uniform than for other experiments, but no observation concerning either the fishing conditions or the experiment itself suggest a cause for this result. (The first observation of mortality in this experiment occurred at 89 hours, the last at 719 hours.) On the other hand, the fifth experiment had a bad start on the fishing vessel. The trawl contents contained unually large numbers of Sea Robins (spiny) and skates (rough skinned). Though fishing had produced ample squid, few survived the journey to the experimental tanks and a reduced number was eventually employed. Compared with similar experiments, mortalities began to appear early, and continued at a high rate for about 100 hours; the last squid was found dead at 306 hours. In spite of contrasts between these five batches, the distribution of mortality was sufficiently broad to mask testable contrasts within *vs.* between them. Mean survival for the five experiments was 218 hours; it was 248 hours if the fifth experiment was thrown out and 262 hours in the last three experiments in which bumper corners were rounded. One-way analyses of variance between all possible pairs of experiments number four, six, seven and eight, were significant (with 95% confidence) only in the contrast between the last two.

The selection of squid for the survival experiments obviously affected the results. An increase in survival from year to year and within experiments conducted in 1971 may be due, in part, to improvements in capture techniques, handling and/or sorting before use. The significant test of the bumper system (in the third experiment) may have been enhanced by utilization of relatively undamaged, seine-caught squid. Poor results in the fifth experiment can probably be attributed to detrimental trawling conditions. Available data do not suggest unusual conditions in the exceptional sixth experiment.

As is the case with most attempts to maintain animals, we were aware of the fact that some squid were becoming "tame." This phenomenon was especially apparent in a pet squid given to us at the end of the summer (1970) by Dr. Ralph Hinegardner. This animal survived 28 days, the last six in our care. It would come to the surface of the tank when gently approached and take both live and killed food from the hand. This animal was a one year old female, and she deposited a number of egg masses in captivity, the last of which were infertile. The Hinegardner squid, like our longer-lived ones, did not so readily race around the tank as "wild" squid, though all of them could be startled. Our long-lived squid overcame transportation to new surroundings with less apparent trauma than most squid exhibit on first being placed in any tanks. All long-lived squid learned to take notice of our activities and seemed to recognize feeding times. No attempt was made to tame the experimental animals, though our observations cause us to suggest that it is possible, realizing that the squid may have the same object in mind.

Damage to the tail end of the captive squid was previously reported (Summers and McMahon, 1974). Bumpers reduced this damage, but introduced a new hazard, because squid would slip under the tubing, getting between it and the tank wall. (We restored all such errant squid to the tank center at each observation.) Though restrictive, this change in location was not usually fatal, and it appeared to us that some smaller squid (especially females) sought it out to avoid other squid. Careful adjustment of the bumper height helped prevent entrance to this narrow space, but no complete solution was found. Rounding of the bumper corners aggravated the problem of adjustment while preventing squid still inside the bumper from their senseless battering in corners. One of us (Ruppert), made preliminary tests on an alternative bumper or divider which was created by a continuous curtain of small air bubbles. Simple experiments in a tank generously provided by Mr. Charles L. Wheeler of the National Marine Fisheries Service (Woods Hole), demonstrated that squid usually would not cross such a barrier even if they were starved and live *Fundulus* (which also respected it) were on the other side. Although the technology of creating a uniform bubble curtain is complex, applications of this device might be suitable for squid maintenance facilities.

Mr. John Aldrich of the Boston University Marine Program (Woods Hole) kindly performed oxygen analyses of seawater in the experimental tanks. Samples were taken at six locations (the inlets to each upper tank and at the centers of the four experimental tanks) on four different dates (August 19 and 24, September 1 and 8, 1971). Seawater temperature ranges were 20.4–21.8° C in the ambient temperature tanks and 15.0–17.4° C in the chilled tanks. The total range of his 24 titration determinations was 4.31 to 5.25 ml of dissolved oxygen per liter, corrected to standard temperature and pressure; these values approach appropriate saturation levels. No consistent trend was measured in the water course of either tank stand, and no consistent difference was observed between stands; the maximum variation within readings from the same data and tank stand was about 0.5 ml/liter. The pH was determined at these same six locations on September 1 and 10, 1971. It was constant at all locations on each date; 8.05 on September 1 and 7.98 on September 10 (seawater temperatures of 15.6 and 21.0° C). No dissolved zinc analyses were made, because effects produced by the galvanized heat exchanger were confounded with relative water temperature, and the latter was not found to be a significant factor.

The apparent anomaly of longer squid survival in the most oblong tanks occurs in the present results as it did in the previous study (Summers and McMahon, 1974). Though not statistically significant in current results, survivorship was longer in the upper tanks. It may be noted that the "rectangular cells" in previous work were located in these same upper tanks because cell shapes were physically nested in tank levels. Without evidence for changes in water quality, we can only assume that these upper tanks are particularly suited for survival.

Squid survival was broken down into components by age, sex, and season because these three were interrelated. Results are graphically presented in Figure 2. Sizes of individuals in the different age groups (which were used in identifying the age groups) were practically the same as those reported for the previous year (Summers and McMahon, 1974). Seasonal occurrence of the age groups was slightly different due to the small sample sizes provided by these experimental animals. As previously observed, two year olds may hold a small survival advantage over one year olds when these are mixed together early in the season. Young-of-the-year squid show a great disadvantage in survival when mixed with one year olds. We also observed that young-of-the-year squid suffered more skin abrasions in the trawl than older age groups and that smaller squid receive a greater amount of abuse from their tank mates.

An apparent trend in survival of one year olds through the season probably reflects changes in experimental design, principally the removal of dividers and universal installation of bumpers, as well as the annual reduction in proportion of sex-

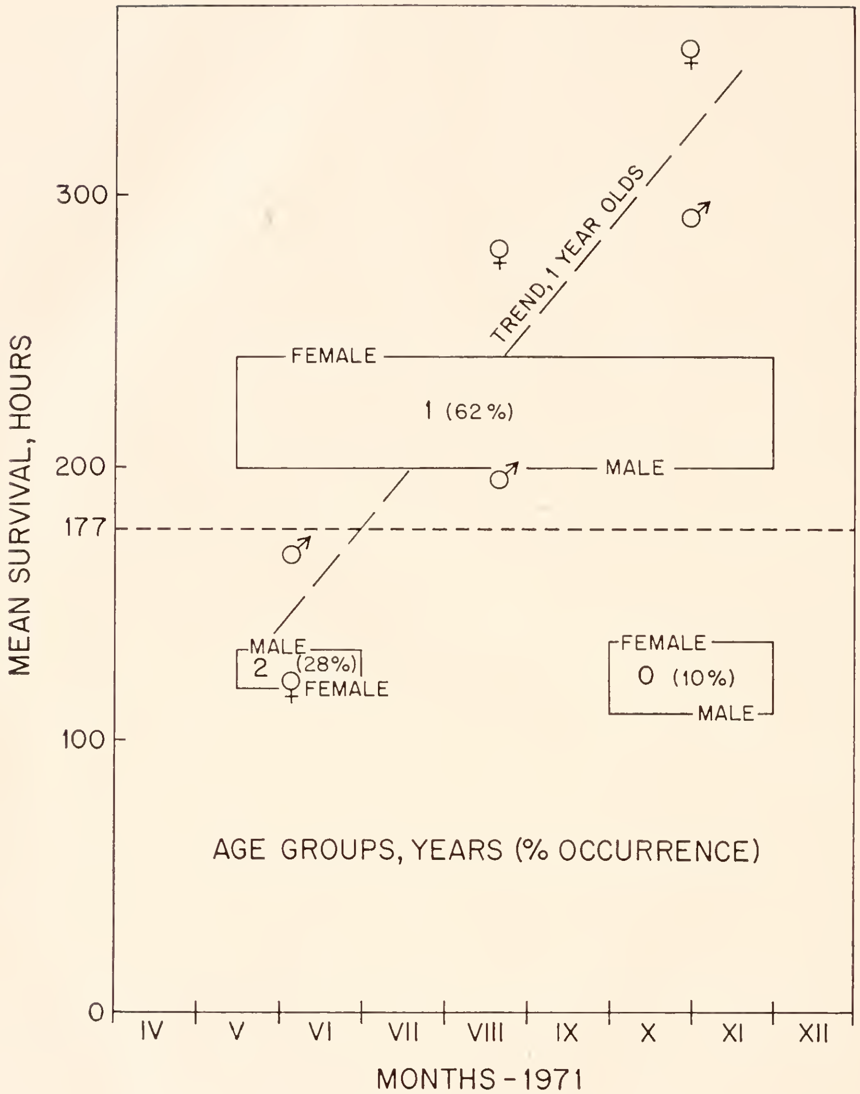

FIGURE 2. Mean survival of squid by age, sex and season. The overall mean, 177 hours, is indicated in the figure. Insets represent the seasonal occurrence of a particular age group in the experiments and the range of survival times for both sexes as designated on the boundaries. Data for one year olds has been broken down into three equal time periods as shown by paired sex symbols in the figure. An apparent trend in the survival of one year olds probably resulted from annual changes in sexual maturity and improvements in experimental technique.

ually mature squid. It is interesting to note that the initial survival advantage for breeding males gave way to a survival advantage for females later in the season, when sexual maturity, breeding and egg deposition were uncommon.

LaRoe (1971) reported maximum survival of *D. plei* of 38 days and maximum longevity of reared *S. sepioidea* of 146 days (the latter had attained a size of 10.5 cm). His conclusions were that both species of loliginids required 30–60% of their body weight in live food (mysids and larval fish) daily and would starve when fed less than 10–15% of their body weight. He favored opaque tanks (the largest of many different sizes were best), timed lighting which included ultra-violet wavelengths and "semi-natural" substrates in the tanks (sand, gravel, rocks and plastic grass). LaRoe kept adult squid in polyester resin impregnated, wooden tanks, of either 1900 or 2100 liter capacity; these had an exchange of filtered, running seawater once every 4–5 hours. He reported high early mortality of newly captured adults and noted the appearance of sores on the posterior mantle tip of long-lived squid. LaRoe placed window screening over the larger tanks to prevent squid from jumping out, and reported adverse reactions from rapid changes in illumination. Our results differ from his in experimental approach, and we suggest that future studies might wish to evaluate both the ultra-violet illumination and bottom substrates used by LaRoe.

Mikulich and Kozak (1971) reported survival times ranging from 23 to 35 days for the oegopsid squid, *Todardoes pacificus,* when held in large concrete tanks. They observed feeding on a variety of food items, and determined daily rations approximately one-quarter of the squid's weight.

The rearing studies carried out by von Boletzky, *et al.* (1971) and by Arnold, *et al.* (1972) dealt with small species (1–3 cm dorsal mantle length) which spend much of the daytime in sand substrates. Both reports list survivals of approximately 200 days with attainment of full sexual maturity. Spawning occurred in three species reared by von Boletzky, *et al.*, with subsequent death of females (in a few days) and males (in a few weeks). They attributed aquarium mortalities to starvation and skin diseases, noting that smaller animals succumb most readily. Arnold, *et al.*, reported cannibalism occurring when dissimilar sizes were placed in the same tank. Recurrent in these rearing studies is comment concerning difficulty in selecting and presenting food organisms and the tendency of squid to starve over a short period of time. Because of differences in size, we do not feel justification in direct comparisons with the above reports; Bidder (1950) reported rapid digestion in several larger species which is in a much shorter time scale than our observed mortalities. We caution against the transfer of maintenance techniques from one group of cephalopods to another without evaluation of individual requirements.

In concluding this study, we are tempted to generalize on experiences gained from studying the (relatively short) laboratory survival of nearly one thousand *L. pealei* (Summers and McMahon, 1970, 1974, and present report) and from communications with colleagues working on related projects. Progress in our own case can be measured from the results shown in Figure 3, which contrasts squid survival of our earliest and latest work. The exponential "decay" in survival experiments of 1969 has become a cumulative lognormal mortality in the latter half of 1971. Both mean and median survival times have been extended four-fold and it is now practical to speak of a reasonably lengthy survivorship. (Among the 120 squid in experiments four, seven and eight of 1971, 24% lived longer than

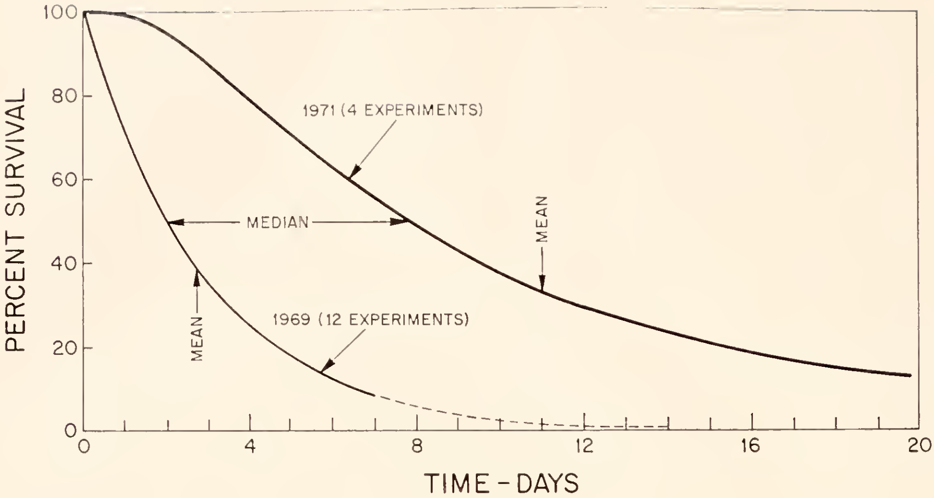

FIGURE 3. Survival models fit to the data from two groups of maintenance experiments. Experiments were truncated at one week in 1969, but the data from approximately 260 squid suggested a proportional mortality with a half-life of two days as shown. A lognormal mortality is indicated for 120 squid in the final experimental design employed in 1971 (the fifth experiment was not included; see text); this extended to 58 days, the record survival time. Improvements in maintenance techniques are apparent in the results both early in the experiments and through the course of the experiments.

two weeks, 12% lived longer than three weeks, 4% lived longer than four weeks, to the record survival of nearly two months.)

Improvements in our initial survivorships were probably due to modifications in the capture techniques. Much room for further improvement exists in this area, though practical considerations will probably always limit the methods employed. Ample water exchange, low disturbance, opaque tanks with lids and regulation in changes of illumination appear to be universally important for the maintenance of non-burrowing squids. Within natural limits, squid seem to tolerate a variety of seawater temperatures and crowding conditions and, feeding regimens. Mortalities resulting from collisions with the tank walls can be minimized by using larger tanks and/or applying bumpers inside the tank walls. Breeding and egg deposition are indicators of subsequent death, usually sooner in the case of females. The simple attainment of sexual maturity may augment mortality, as witnessed by several truncated rearing experiments (Choe, 1966; LaRoe, 1971; Arnold, *et. al.*, 1972). Thus, obtaining virgin individuals or sexual isolation may be of little value. Sorting for age groups (perhaps, simply by size) will probably aid in promoting survival. Rearing of species through complete life cycles will be most readily accomplished in those cases where the eggs are large and the fry are well developed (*L. pealei* does not possess these advantages). As stressed by LaRoe (1971), the selection of food types will be a problem in many cases.

The authors wish to acknowledge the considerable help given them by numerous colleagues and the staff of the Marine Biological Laboratory. Drs. H. Burr Steinbach and Edward F. MacNichol were especially instrumental in stimulating this project. Several friends and associates were made to perform hard physical work, log data and sort through trawl catches in exchange for day trips aboard the fishing vessels, most durable among whom was Ms. Eleanor Lipshitz. Other colleagues provided comments and suggestions on call and endured our obsession with squid. We especially wish to cite Captain Henry Klimm and the personnel of the National Marine Fisheries service in this respect. Captains Peter Graham and David Graham and their respective crews were very helpful in this project. Drs. John M. Arnold and David E. Schneider provided useful criticisms of both manuscripts.

SUMMARY

1. This paper reports continued experimentation on factors affecting the survival of the squid, *Loligo pealei,* in laboratory aquaria. A total of 246 squid were studied in eight separate experiments which were run between May 18 and December 4, 1971.

2. Various tank sizes and configurations were studied in conjunction with evaluations of a tank wall bumper system and relative water temperature. Uncontrolled factors such as ambient seawater temperature, age, sex and sexual maturity were studied over the course of the experiments.

3. Mean survival of all squid was 177 hours and maximum survival was 1400 hours. In the final design, the mean survival was 248 hours (120 squid). Mortalities in this last group closely followed a log-normal model. The bumper system was found to produce significant survival advantage (with better than 99% confidence) through an analysis of variance of one experiment.

4. The "state of the art" is discussed with some generalizations and suggestions for further investigations.

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THE VANADIUM AND SELECTED METAL CONTENTS OF SOME ASCIDIANS

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The concentration of vanadium and other metals from sea water by animals called ascidians has been known since the turn of the century. Several papers have summarized the information available on the subject (Webb, 1939; Bertrand, 1950; Carlisle, 1968). This paper reports the vanadium and other selected transition metal contents of ascidians primarily from the northern California coast. It has been suggested that the ability to concentrate vanadium to levels greater than the combined levels of other transition metals is confined to certain families in the order Phlebobranchia (Webb, 1939; Carlisle, 1968), but several workers have reported finding substantial concentrations of vanadium in species from the order Aplousobranchia (Goldberg, McBlair and Taylor, 1951; Trason, 1957; Levine, 1961; Ciereszko, Ciereszko, Harris and Lane, 1963). Studies were carried out on certain species from the order Phlebobranchia and Aplousobranchia to determine vanadium content. In addition, since large iron contents have been reported (Endean, 1955) for species from the order Stolidobranchia, observations were made on a species from this order. Electron spin resonance studies were carried out to determine the oxidation states of vanadium in various species. Preliminary spectra are reported for the cells contained in the fluids of *Ascidia ceratodes* and the fractions chromatographed from these cells.

MATERIALS AND METHODS

Species were collected at the following locations in the spring and summer of 1972 and the spring and summer of 1973: Bodega Bay, *Ascidia ceratodes;* Bodega Marine Laboratory, University of California, *Eudistoma psammion* and *Eudistoma ritteri;* Berkeley Yacht Harbor, *Ciona intestinalis* and *Molgula manhattensis;* Hopkins Marine Station, Pacific Grove, *Amaroucium californicum, A. solidum, Clavelina huntsmani, Cystodytes lobatus, Distaplia occidentalis,* species of genus *Eudistoma, Polyclinum planum* and *Synoicum par-fustis. Rhopalaea abdominalis*, a Caribbean species collected south of Portobello, Panama by Dr. Charles Birkeland, was supplied as a preserved sample by Professor Donald P. Abbott, Hopkins Marine Station. The samples collected at Hopkins Marine Station were identified by Professor Donald P. Abbott. Other samples were identified by comparison with descriptions in standard references (Van Name, 1945; Ricketts, Calvin and Hedgpeth, 1968).

Each sample was cleaned of extraneous material and colonial ascidians were cut to minimize the contamination of the sample by sand. Solitary ascidians were brushed clean and washed. The samples were weighed wet, after drying at 110° C and after ashing at 500–600° C to constant weight. The ashes of several colonial ascidians were redissolved in aqua regia, any remaining particulate matter was removed by filtration and the samples were redried. Such a procedure has no effect on the vanadium analyses. However, there was a direct correlation between the spectrographically determined silicon content of the sample and the iron content. This may result from either the inclusion of sand or ingested diatoms, some of which are known to concentrate iron, in the ascidians. Therefore iron contents are reported only for those samples that have low silicon contents.

The spectrographic analyses were run on a Bausch and Lomb Large Littrow-type Quartz Prism Spectrograph. To insure a proper arc both samples and standards were mixed 1 : 1 by weight with a buffer consisting of (by weight) 50% graphite, 25% $Al_2(SO_4)_3$, and 25% K_2SO_4. Prior to use the graphite electrodes were refluxed in concentrated nitric acid for 2 days. The spectra were recorded on Eastman SA-1 plates. The plates were read with an Applied Research Laboratory Model No. 42 Densitometer. The following lines were used for quantitative determinations of the elements: vanadium, 2526.22 Å, 2530.18, 2923.62, 2924.03, 2924.64, 3183.41, 3183.98 and 3185.39; titanium, 3186.45; chromium, 2677.16 and 3015 (3 lines); iron, 2689.21, 2714.87, 2719.03, 2720.91, 2723.57, 2724.9 (2 lines) 2733.58 and 2735.48; and silicon, 2519.21 and 2528.54. The concentrations of the metals were determined by comparison of the observed density of the line after correction for background with a series of standards. Spectrophotometric analyses using a Cary Model 14 Spectrophotometer were performed for vanadium using the phosphotungstate (Wright and Mellon, 1937) and N-benzoyl-o-tolylhydroxylamine methods (Majumdar and Bhowal, 1971) using ammonium metavanadate as a standard. Iron analyses were performed using the thiocyanate method (Sandell, 1959). The approximate pHs of the samples were determined with universal pH indicator paper. Electron spin resonance (e.s.r.) spectra were recorded at 77° K on a Varian Model E-4 Spectrometer. Order of magnitude concentrations of vanadium (IV) were determined by comparison of the intensities of the spectra with standard solutions of known concentrations run at 77° K.

Spectra of the cells from *Ascidia ceratodes* were taken by allowing them to adhere to the window of a quartz cell and then the spectra were recorded with a Cary Model 14 spectrophotometer equipped with a Model 1471250 High Intensity Light Source. A separation of the contents of the cells in the fluid from *Ascidia ceratodes* using column chromatography is reported. The separation was carried out on CPG-10-175 Controlled-Pore glass beads coated with Carbowax, mesh size 80/120, manufactured by Electro-Nucleonics Inc., using a thermostatable column manufactured by Pharmacia Fine Chemicals, Uppsala, Sweden. Oxygen was purged from the elutent, 0.1 M H_2SO_4, with nitrogen, which had been purified by passing the gas through chromium(II) bubblers. The samples were collected, 4-cc per collection, with a Gilson Automatic Collector and care was taken to flush the collection tubes with nitrogen to minimize oxygen oxidation of the samples. The sample to be eluted was prepared by the following steps: (1) the fluid was centrifuged and the plasma removed; (2) a minimal quantity of 0.1 M H_2SO_4 was added to the cells and the cells were lysed and (3) the residue was centrifuged away from the green-yellow solution. All operations were performed in a nitrogen atmosphere to minimize air oxidation. Protein analyses were made with Folin reagent.

TABLE I

Transition metal analyses of ascidians

Species and systematic position	ppm (org dry wt)[a]		wet weight, g	dry wt % of wet wt.	ash wt % of wet wt.	Comments
	V	Fe				
PHLEBOBRANCHIA						
ASCIDIIDAE						
Ascidia ceratodes						
whole animal	1300	1900	8.46	7.80	2.72	—
blood (ash)	6200	500				$[\mathrm{V}] \approx 3\text{–}4 \times 10^{-3}$ M,[c] acid, no e.p.r. signal
outer fluid (ash)	8000	500				
fluid[b]	—	—	—	—	—	$[\mathrm{V}] \simeq 2 \times 10^{-3}$ M
CIONIDAE						
Ciona intestinalis						
blood (ash)	160	50	—	—	—	$[\mathrm{V}] \approx 7 \times 10^{-5}$ M[c] Mn 10 ppm
centrifuged blood cells	—	—	—	—	—	$[\mathrm{V(IV)}] \approx 3 \times 10^{-4}$ M[d]
PEROPHORIDAE						
Perophora annectens						
whole animal, 1972	8000, 9000[b]	940	4.85	7.24	3.51	acid, no e.p.r. signal
whole animal, 1973	700–2000[h]	—	—	—	—	
DIAZONIDAE						
Rhopalaea abdominalis						
whole animal, preserved	1800	—	5.88	3.97	2.17	
APLOUSOBRANCHIA						
SYNOICIDAE						
Amaroucium californicum (ash)	<10	[e]	3.04	—	5.34	
A. solidum (ash)	<10	~1000	4.78	—	2.94	
Polyclinum planum (ash)	<10	[e]	10.82	10.80	7.55	
Synoicum par-fustis	~10	600	18.81	6.20	2.90	
POLYCITORIDAE						
Clavelina huntsmani	30	900	19.36	3.91	2.15	—
Cystodytes lobatus	50	[e]	5.18	—	6.48	—
Distaplia occidentalis whole colony						
1. orange-yellow	600	1200	2.17	6.0	2.8	acid, pH ~1
2. light green-brown-gray	1200	2200	2.96	6.0	3.0	acid, pH ~1
3. brown red-light brown	600	500	2.79	6.0	2.6	acid, pH ~1
4. brown-white-light brown	1200	2200	3.00	6.0	3.0	acid, pH ~1
Eudistoma diaphanes						
whole colony	25	[e]	4.97	6.60	2.90	acid, pH ≤2

TABLE 1—*Continued*

Species and systematic position	ppm (org dry wt)[a]		wet weight, g	dry wt % of wet wt.	ash wt % of wet wt.	Comments
	V	Fe				
E. molle						
whole colony surface including zooids	1000	400	2.90	5.90	2.20	acid, pH ≤2
zooids	560–1200[h]	—	—	—	—	—
zooids (ash)	6000, 5000[b]	—	—	—	—	—
test (ash)	1000[b]	—	—	—	—	—
E. psammion						
whole colony	80	e	13.28	16.5	11.6	acid, pH ≤2
whole colony	130[h], 130[h]	—	11.30	6.82	3.06	—
zooids	610[b], 614[h]	—	0.160	17.60	3.39	—
E. ritteri						
large whole colony (ash)	320	2600	1.06	—	3.54	Cr, 50 ppm; Ti, ~150 ppm
small whole colony (ash)	270	2400	0.41	—	4.72	Cr, 60 ppm; Ti, ~100 ppm
STOLIDOBRANCHIA						
MOLGULIDAE						
Molgula manhattensis						
animal without tunic-intestines cleared	—	1600[f]	1.19	5.00	2.51	
	—	900[f]	1.67	4.09	2.07	
	—	1200[f]	1.25	4.10	2.19	
	—	1700[f]	1.17	3.95	2.20	
whole animal without tunic-fluid removed from heart-intestines cleared	—	1300[f]	0.716	4.98	2.14	
fluid from heart-intestines cleared	—	8000[f]	0.2015[g]	3.34	2.68	
	—	5000[f]	0.2365[g]	3.60	2.43	
fluid from heart-freshly collected	—	5700[f]	0.2435[g]	3.30	2.25	
animal without tunic-freshly collected	<20	16,400	2.21	6.21	3.60	
	—	17,500[f]	2.02	6.49	3.52	
	—	26,600[f]	3.17	5.92	3.67	
whole animal	<20	—	3.67	6.0	3.0	

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[a] ppm (parts per million) organic dry weight unless otherwise specified (*e.g.*, ash-indicates ppm ash weight). Organic dry weight = dry weight—ash weight.

[b] Vanadium determined spectrophotometrically using phosphotungstate method.

[c] "Vanadium concentration" determined from analysis of a known volume of fluid.

[d] Vanadium (IV) concentration estimated using e.s.r. standardized against a 1×10^{-3} M vanadium (IV) in 0.5 M $HClO_4$ at 77° K.

[e] Analysis for Fe unreliable due to presence of particulate matter.

[f] Analysis for Fe using thiocyanate method.

[g] Wet weight is weight of fluid used.

[h] Vanadium determined spectrophotometrically using o-benzoyl-o-tolylhydroxylamine.

RESULTS AND DISCUSSION

Observations on species from the order Phlebobranchia

Table I contains data on the vanadium, iron and selected transition metal contents of various ascidians. The species are arranged alphabetically within the orders Phlebobranchia, Aplousobranchia and Stolidobranchia respectively. The species *Ascidia ceratodes* and *Perophora annectens* in the families Ascidiidae and Perophoridae of the order Phlebobranchia have large vanadium contents. These observations are consistent with analyses performed on other species in these two families (species, ppm V in whole animal based on organic dry weight): *Ascidia corelloides*, 1130; *A. nigra*, 3100; *A. mentula* forma *cylindrica* Harant, 470, 1400; *A. mentula* forma *typica* O. F. Muller, 420, 610, 982; *A. aspersa* forma *cristata*, 690, 790; *Phallusia mamillata*, 1200–1300, 1700; *Perophora viridis*, 760; *Ecteinascidia conklini*, 1800; and *E. turbinata*, 100 (work of Webb 1939; Bertrand, 1950; Ciereszko, Ciereszko, Harris and Lane, 1963; Carlisle, 1968).

It should be noted that vanadium analyses of *Perophora annectens* varied between 700–2000 ppm organic dry weight in the spring and summer of 1973 and 8000–9000 ppm in the spring and summer of 1972. It may be that the difference can be ascribed to the maturity of the samples, but no specific study was carried out on this interesting subject (see discussions of *Eudistoma molle*, *E. psammion* and *E. ritteri*). Samples of *Ascidia ceratodes* collected during both periods had identical vanadium contents in their fluids. Known volumes of the fluids from the heart and the region just inside the tunic of *Ascidia* ceratodes were reduced to ash, analyzed and "vanadium concentrations" of the fluids were calculated to be between 2–4 × 10^{-3} M. The homogenized fluids of both *Ascidia ceratodes* and *Perophora annectens* were acidic and initially showed no electron spin resonance (e.s.r.) spectrum at 77° K. At later stages the e.s.r. spectrum of vanadium (IV) developed. These observations are consistent with those made on fluids present in the species *Phallusia mamillata* (Boeri and Ehrenberg, 1954; Bielig, Bayer, Dell, Rohns, Mollinger and Rüdiger, 1966), and *Ascidia obliqua* (Boeri and Ehrenberg, 1954; Lybing, 1954), where it is suggested that vanadium (III) is present. It should be noted that in the case of *Ascidia ceratodes* the iron content of the whole animal is considerably higher than the vanadium content, while in the fluids the vanadium content is at least ten times that of iron. *Ciona intestinalis* also shows the property that the blood cells contain a large excess of vanadium over iron while the metal content of the whole animal shows the reverse (Bielig, Pfleger, Rummel and Seifen, 1961). The surface of the tunic of *Ascidia ceratodes* was carefully cleaned to eliminate extraneous matter as a source of iron.

A chromatographic separation of the contents of the cells contained in the fluid of *A. certaodes* was carried out using the methods described under Materials and Methods. Figure 1 represents the spectral characteristics of the dominant species collected and of the cells in a plasma-saline solution. The number of the fraction is used to label a particular spectrum. Fraction 26 and those having the same spectral characteristics contained all of the vanadium in the sample. The vanadium content of a control sample containing the same volume of fluid was 9.8 × 10^{-3} millimoles, while 9.9 × 10^{-3} millimoles were accounted for by analysis of the vanadium in fractions 26–29. The spectrum of fraction 26 can be reproduced by a combination of the spectra of vanadium (III) and vanadium (IV) in 0.1 M H_2SO_4,

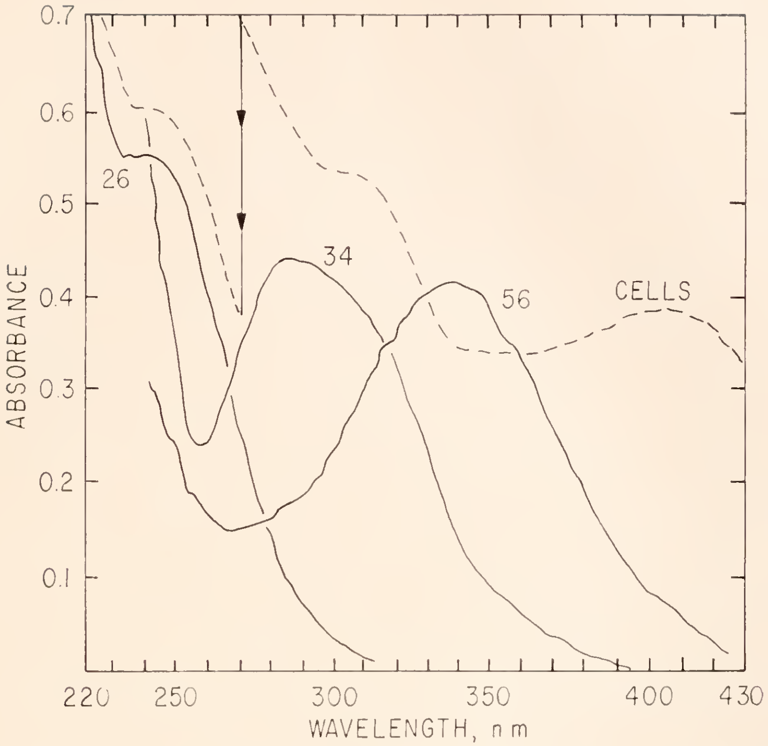

FIGURE 1. Spectra of chromatographed fractions and cells of *Ascidia ceratodes.*

and no spectral evidence is found for a ligand or protein bound to the vanadium. A vanadium (III)-vandium (IV) mixture in 0.1 M H_2SO_4 was passed through the column under the same conditions as the sample and it has the same fraction distribution as the sample. Electron spin resonance (e.s.r.) and spectral studies show some vanadium (IV) is present in the eluted fractions. Therefore it appears that once the cells are lysed some oxidation of vanadium (III) occurred even though precautions were taken to exclude oxygen. Samples eluted without the precaution of removing oxygen showed more vanadium (IV) than those where oxygen was excluded. We feel a possible role of vanadium (III) in the cells is the production of acid by means of an oxidation-reduction cycle.

Using Folin reagent the protein contents of various fractions were (fraction number: μg/ml protein): 26:5, 33:19, 34:33, 38:7 and 56:0. The amount of protein seems to vary with the absorbance represented by fraction 34, which is approximately the absorbance maximum of tryptophan, which has been shown to be present in the protein of *Phallusia mamillata* (Bielig, Bayer, Dell, Rohns, Mollinger and Rüdiger, 1966).

Fraction 56 contains a compound with absorbance maxima at 335 and 205 nm. Aqueous solutions containing this compound (although not shown to be pure) obey Beer's law and are oxidized by vanadium (V) and oxygen. It is not clear what role this species plays in the *in vivo* system, but the possibility is being investigated that cells containing this compound are involved in the reduction of vanadium (V), the oxidation state of vanadium present in sea water, to the vanadium (III) present in the cells.

As shown in the spectrum of the cells, the components represented by fractions 26 and 34 can account for the shorter wavelength region, but the species represented by fraction 56 appears to be shifted to long wavelengths. Ethanol solutions of fraction 56 are shifted to longer wavelengths and the cell spectrum may reflect the environment of the species in fraction 56.

The vanadium content determined for a preserved sample of *Rhopalaca abdominalis* (1800 ppm organic dry weight) was comparable to that determined for *Rhopalaca neapolitana*, 1700, 2000 ppm organic dry weight (Ciereszko, Ciereszko, Harris and Lane, 1963). The preservation fluid also contained some vanadium suggesting that the metal can be leached from the animal. Thus the value of 1800 ppm is a lower limit on the vanadium content of *Rhopalaca abdominalis*.

The vanadium content of *Ciona intestinalis* is in agreement with the observations of other workers (Goldberg, McBlair and Taylor, 1951; Bielig, Pfleger, Rummel and Seifen, 1961; Carlisle, 1968). The vanadium and iron contents of the blood cells have been determined to be 1500 and 800 ppm dry weight respectively (Bielig, Pfleger, Rummel and Seifen, 1961). The values determined in this study of 160 and 50 ppm ash weight for vanadium and iron in the blood seem reasonable. The manganese content of blood is less than 10 ppm. The high level of manganese found by Carlisle (1968) must result from localization of the metal in other areas of the animal. The 77° K electron spin resonance spectrum of cells centrifuged from the blood of *Ciona intestinalis* shows the presence of vanadium (IV). If the intensity of the e.s.r. spectrum is compared with those of standard solutions and the resulting vanadium (IV) concentration is compared with the vanadium content obtained by direct spectrographic analyses of the blood, it appears that a substantial amount of the vanadium in the blood is present as vanadium (IV) or in a vanadium oxidation state which is readily oxidizable to vanadium (IV).

Observations on species from the order Aplousobranchia

Large vanadium contents have normally been associated with species of certain families of the order Phlebobranchia. However, large vanadium contents and acidic conditions have been found in certain species in the order Aplousobranchia: *Euherdmania claviformis, Amaroucium pellucidum, Eudistoma olivaceum, Eudistoma ritteri, Clavelina picta*, and *Pycnolclavella stanleyi* (work of Goldberg, McBlair and Taylor, 1951; Trason, 1957; Levine, 1961; Ciereszko, Ciereszko, Harris and Lane, 1963). Our investigation shows that certain species in the genera *Distaplia* and *Eudistoma* in the family Polycitoridae from the order Aplousobranchia concentrate large amounts of vanadium. All of the species were acidic, pH ≤ 2. A high level of sulfuric acid in the tunic fluid of *E. ritteri* was observed by Levine (1961). The species with large vanadium contents were *Distaplia occidentalis* (four separate color variations) and some species in the genus *Eudistoma: E. molle* and *E. psammion*. *E. molle* and *E. psammion* were examined to determine the location of the vanadium (zooids or distributed throughout the entire colony). The orange zooids of *Eudistoma molle* were carefully removed from the animal and both the zooids and test were analyzed for vanadium. On the basis of concentrations in ppm ash weight at least 80% of the vanadium present is localized

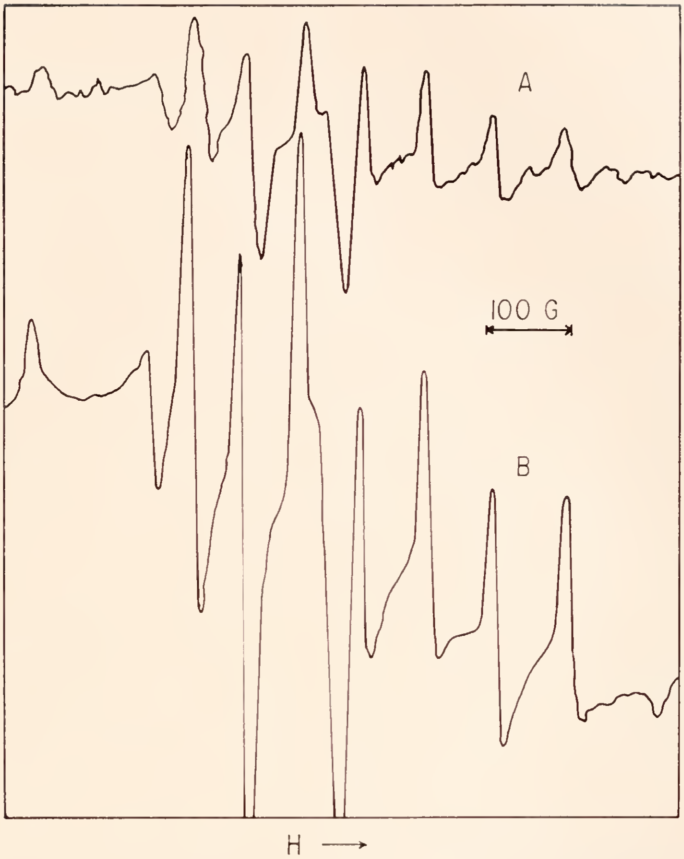

FIGURE 2. Electron spin resonance spectra at 77° K of *Eudistoma diaphanes* (A) and 10^{-2} M VO^{2+} in aqueous acid (B).

in the zooids. Some variation occurred in the vanadium contents of zooids of samples collected in 1972 and 1973, and it is believed that less mature animals contained less vanadium in the zooids. The vanadium in *E. psammion* is also localized in the zooids and analyses in 1972 and 1973 showed identical vanadium contents for whole animals and zooids. The low vanadium content determined for *E. diaphanes*, 25 ppm organic dry weight, may result from the low density of zooids in this species compared to other species of this genus. Levine (1961) has determined the metal content of the zooids from *E. ritteri* and has found the following metals in ppm dry weight: Ti, 1512; V, 471 and Cr, 144. Our analyses in ppm ash weight of *whole animals* show vanadium and chromium at the expected levels, but titanium is an order of magnitude below the level reported by Levine. We have observed variable levels of some metals in *E. ritteri*, for example, some very immature colonies showed a lower vanadium content than more developed colonies. It is possible that animals at various stages of development require different metals, and consequently some discrepancies in metal analyses appear. The metal content of the marine environment just before and/or at the time of sampling may be important.

The question of the oxidation state of the vanadium present in these species arises. Figure 2 shows the e.s.r. spectrum of vanadium (IV), as VO^{2+}, in an aqueous acid solution at 10^{-2} M and the colonial tunicate *E. diaphanes* at 77° K. Similar spectra were observed for *E. molle*, *E. ritteri* and *E. psammion*. The spectra recorded are at different sensitivities and are shown to confirm that vanadium (IV) can be detected in these samples. On the basis of comparisons with standards of known vanadium (IV) concentrations a major fraction of the vanadium present is in the plus-four oxidation state. Care was taken to protect the samples from oxygen, but it cannot be ruled out that the vanadium (IV) was produced from the rapid oxidation of a complex containing vanadium in a lower oxidation state. Although vanadium (III) is only slowly oxidized by oxygen in aqueous, acid solution (Ramsey, Sugimoto and De Vorkin, 1941), certain vanadium (III) complexes in organic solvents (Swinehart, 1971) and aqueous vanadium (II) (Swinehart, 1965) are rapidly oxidized by oxygen. However, the vanadium (III) complexes present in *Phallusia mamillata* (Bielig, Bayer, Dell, Rohns, Mollinger and Rüdiger, 1966) and *Ascidia obliqua* (Lybing, 1954) under acid conditions are slowly oxidized by oxygen. This type of vanadium (III) complex can not be a precursor to the observed vanadium (IV) complex. The question remains as to whether the acid and vanadium are localized in the same cells. Levine (1961) has shown that the "green-cell" blood corpuscles of *Eudistoma ritteri* do not contain acid and do not reduce osmic acid; the latter is a test for the vanadium (III) complexes found in the blood of certain species. Our observations are consistent with the conclusion that vanadium is in the plus four oxidation state in species of the genus *Eudistoma* and consequently may not be susceptible to oxidation by osmic acid.

Observations on a species from the order Stolidobranchia

All other ascidians studied had small vanadium contents or large iron contents compared to other metals present. A study by Carlisle (1958) on the metal content of pooled samples of *Molgula manhattensis* from the order Stolidobranchia shows the presence of vanadium (101 ppm dry weight of flesh without tunic) and niobium (56 ppm dry weight). The vanadium content in this study of the animals without tunic is less than 20 ppm organic dry weight, which is substantially below the level previously reported. This discrepancy may actually be a result of species differences (Monniot, 1969). The iron content of the animal without tunic is 900–1700 ppm organic dry weight. It was noted that freshly collected animals exhibited very large iron content (16,400–26,600 ppm organic dry weight) compared to animals which were kept in a salt water aquarium for several days (900–1700 ppm organic dry weight). The large iron content observed for freshly collected specimens arose from ingested particulate matter from the collection area. A large percentage of the iron in the animals (kept in a salt water aquarium) is localized in the fluid from the heart and the amount of the iron in heart fluid is the same for both freshly collected and aquarium specimens.

Metal contents and evolution of ascidians

Millar (1966) has suggested that the order Aplousobranchiata, Aplousobranchia according to Van Name (1945), be divided into three families. One of the families, Clavelinidae, is divided into three subfamilies: Clavelininae, Holozoinae and Polycitorinae. The two most developed subfamilies are Holozoinae, which contains the genus *Distaplia*, and Polycitorinae, which contains the genus *Eudistoma*. It seems probable that the adaption of species in the genera *Distaplia* and *Eudistoma* to vanadium occurred independently of species in the order Phlebobranchia and consequently the difference in the oxidation states of vanadium in the different orders is not surprising since the functions of the metals may be different.

It has also been suggested (Webb, 1939) that an early ascidian form had the ability to accumulate vanadium at the expense of other metals, the ability to accumulate vanadium was lost in the two evolutionary lines that developed from this form, and these two evolutionary lines and the accompanying loss of vanadium culminated in the iron containing species of the order Aplousobranchia (*e.g.*, *Amaroucium californicum, A. solidum, Polyclinum planum* and *Synoicum par-fustis*) and Stolidobranchia (*Molgula manhattensis*). Along these evolutionary lines there could be transitional species which have the ability to accumulate both iron and vanadium. The iron contents of *Eudistoma molle* and *Distaplia occidentalis* are of the same order of magnitude as the vanadium. It is possible that these species represent animals which are in transition between the vanadium and iron users.

The authors thank Professor Donald P. Abbott, Hopkins Marine Station for his untiring interest in the problem. Extremely useful comments by an unknown, invertebrate zoologist referee are also acknowledged. Research support is acknowledged from the Research Corporation and Petroleum Research Fund administered by the American Chemical Society (PRF-6686-AC3). Spectrographic analyses were in part carried out by John Voth and the e.s.r. spectra were recorded by Dr. Ted Richerzhagen.

SUMMARY

The vanadium and other selected metal contents of primarily California ascidians have been determined. The species *Ascidia ceratodes* and *Perophora annectens* have large vanadium contents as has been predicted for members of the families Ascidiidae and Perophoridae from the order Phlebobranchia. Several species in the order Aplousobranchia have large vanadium contents: the vanadium being present as vanadium (IV) whereas it is vanadium (III) that is found in the order Phlebobranchia. *Molgula manhattensis*, a species from the order Stolidobranchia, shows a large iron content: the metal being localized in the fluid from the heart.

Three dominant fractions were chromatographed from the cells contained in the fluid of *Ascidia ceratodes*. The roles of the compounds present in these fractions are discussed. The spectra of these fractions are correlated with the spectrum of the cells.

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EFFECT OF THE POTASSIUM ION ON INDUCTION OF NOTOCHORD FROM GASTRULA ECTODERM OF *RANA PIPIENS* [1]

Reference: *Biol Bull.*, **146**: 313–325. (June, 1974)

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Small aggregates of cells prepared from explants of ventral ectoderm of *Rana pipiens* gastrula can be induced to differentiate into a variety of cell types by altering the ionic composition of the medium in which they are treated or cultured (Barth and Barth, 1959, 1969). For example, nerve of two distinct types and pigment cells appear in experimental cultures, while epithelial sheets with ciliated patches or free-swimming ciliated masses differentiate from untreated control cell aggregates. These several types of differentiation are the end result of a sequence of steps beginning with an initial treatment period of 2–3 hrs, followed by a Na-dependent reversible period of 4–6 hrs and a final period of 5 or more days before visible differentiation (Barth, 1966; Barth and Barth, 1969).

The cations used in these studies (Na^+, K^+, Ca^{2+}, and Mg^{2+}) are supplied at concentrations well below the level where osmotic shock and cell injury occur (Holtfreter and Hamburger, 1955, page 273). The concentrations used can be applied continuously and the cells will live and differentiate. Thus we are working *in vitro* within a range of cation concentrations that could occur at local regions within the whole gastrula. The working hypothesis has been that during the course of normal gastrulation and later differentiation, release of cations from bound or sequestered states, followed by binding at new sites within the embryo, leads to conditions similar to those provided in cell cultures and found effective in switching cells to a new type of differentiation (Barth and Barth, 1974).

In addition to the naturally occurring cations, we have used the lithium ion as a tool for bringing about the initial step in induction. In the experiments reported below gastrula ectoderm cells were treated for 2–3 hrs in standard solution to which 71 mM Li^+ was added. Following this treatment the cells regularly differentiate into nerve and pigment cells when cultured in standard solution containing 1.3 mM K^+. In the course of experiments elsewhere reported (Barth and Barth, 1974) it was discovered that increasing the concentration of K^+ in the culture medium causes lithium-induced ectoderm cells to differentiate into notochord. The purpose of

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the present paper is to describe and discuss these experiments and their implications in relation to ionic regulation of cellular differentiation.

MATERIAL AND METHODS

Eggs of *Rana pipiens* obtained by pituitary injection were used at mid-gastrula stage (S11, Shumway, 1940). The basic methods of obtaining the cells used for experiments and the composition of standard solution have been published earlier (Barth and Barth, 1959 and 1969) and have been summarized more recently (Barth and Barth, 1974).

The cells

Small explants consisting of approximately 125 cells were prepared from larger explants of prospective epidermis of the gastrula (ventral ectoderm). The outer pigment coat layer of the large explant was loosened by means of brief treatment with EDTA, peeled off and discarded. The inner layer of ectoderm cells was teased into small explants by means of hair loops. The aggregates of cells thus obtained are small enough to permit ready access to treating solutions without having been subjected to complete dissociation and possible damage to cell surfaces that isolated embryonic cells at this early stage might suffer from the EDTA solution.

Six large explants yield approximately 150 small explants containing about 125 cells per explant. These cell aggregates were treated with test solutions in 15 ml stender dishes and are cultured on coverglasses in 5 ml stender dishes. More than 5,000 small explants, or cell aggregates, were used in the experiments reported presently.

Permanent preparations of cells adhering to coverglasses are made by washing out the culture medium with standard solution lacking serum and replacing with Bouin's fluid for a minimum of 120 min. Bouin's solution is replaced by successive tap water rinses and the preparations are stained for 10 min in Ehrlich's hematoxylin (50% in distilled water). The coverglasses with adhering cells are passed through increasing concentrations of EtOH into 2.5% Eosin Y in 100% EtOH for 1–2 min. Dehydration in 100% EtOH and clearing in xylene require 2–3 min in two changes of each solution. The coverglass preparations are inverted and mounted on glass slides for observation and photographing.

Standard solution

The medium used for operation and culture is a modification of both Holtfreter's and Niu-Twitty's solutions (Barth and Barth, 1959, 1969). This standard solution is varied in the present experiments to contain increasing amounts of K^+. The final solution is prepared from three component solutions made with doubly glass distilled water. *Component A:* NaCl (Biological Grade), 5.150 gr; KCl, 0.075 gr; $MgSO_4 \cdot 7\ H_2O$, 0.204 gr; $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.080 gr; $CaCl_2 \cdot 2\ H_2O$ 0.060 gr; H_2O to 500 ml. *Component B:* $NaHCO_3$, 0.200 gr; H_2O to 250 ml; *Component C:* Na_2HPO_4, 0.0300 gr; KH_2PO_4, 0.375 gr; H_2O to 250 ml. The final solutions are prepared by mixing aliquots of 50 ml of A, 25 ml of B, and 25 ml of C. 100 mg of calf serum (Nutritional Biochemical Corporation) were added to B before mixing. When K^+ was varied, calculated amounts of a standard solution of KCl were added to C before autoclaving and mixing the components. The pH of standard solution is 7.9–8.1. The ionic composition of standard solution expressed in terms of mM is: 90.8 mM Na^+ (2.8 mM from buffers); 1.3 mM K^+; 0.83 mM Mg^{2+}; 0.74 mM Ca^{2+}.

EDTA solution

As used in our system EDTA loosens the outer from the inner layers of prospective epidermis cells, but does not dissociate the gastrula into individual cells. The concentration of EDTA is 10 mg/100 ml of calcium- and magnesium-free buffered salt solution (Barth and Barth, 1959, 1969).

Sterile solutions, glassware and operating instruments are used throughout, and operations are made within a sterile operating cabinet.

TABLE I

Effect of external K^+ on induction of notochord in gastrula ectoderm cells

Concentration of K^+ mM added to standard solution	Types of cellular differentiation
1.3	Radial and ganglionic nerve + pigment cells (1–2)
2.3	Radial and ganglionic nerve; pigment cells (1)
3.3	Pigment cells (1–2); networks (2); notochord + radial and ganglionic nerve (2)
4.3	Networks (2); notochord (2); nerve + pigment cells (1)
5.3	Networks + notochord (3); radial nerve (1); pigment cells (1)
7.3	Radial and ganglionic nerve; pigment cells (1)
9.3	Radial and ganglionic nerve (3); pigment cells (1) to rare

RESULTS

Effect of external K^+ concentration on induction of notochord

Three of the types of cellular differentiation listed in Table I have been photographed and described earlier (Barth and Barth, 1959, 1962, 1963). Nerve appears in cultures in two principal forms. Radial nerve consists of a central mass of neuroblasts from which long nerve fibers radiate outward. Ganglionic type nerve results when neuroblasts migrate outward and accumulate in depots connected by a complex network of nerve fibers. Pigment cells develop from aggregates that first spread out peripherally in a flat, thin sheet and then separate to become single cells. A granular pigmented ring appears next to the nucleus and by about five days of culture the cells become dendritic. Typical pigment cells develop melanin and may live for several weeks in our non-nutrient culture medium. Radial nerve, ganglionic nerve and pigment cells are induced in that sequence, as intensity of the treatment used to bring about the shifts of differentiation is increased (concentration of ionic inductor or length of treatment period), as reported in Barth and Barth (1964).

The new cell type introduced in Table I is notochord, which was recognized when the extensive networks of cells mentioned in Table I were observed to evolve into masses of highly vacuolated cells. The gradual transformation of networks of pre-notochord cells into recognizable masses of notochord cells will be described, together with photographs, in section 2 of the present "Results."

For the experiments summarized in Table I, aggregates of cells prepared from explants of ventral ectoderm were treated with 71 mM Li^+ in standard solution for 2.5–3 hrs. Following this treatment the aggregates were cultured in standard solution in which K^+ was varied from 1.3 mM–9.3 mM. Cultures were observed through 10–20 days; 5.3 mM K^+ is shown to be optimal for notochord differentiation. The table is a summary of results obtained from approximately 5,000 cell aggregates, each containing about 125 cells. Numerals in parentheses indicate intensity of induction of a given cell type as (1) sparse, (2) extensive, or (3) very extensive.

Table I shows that at 1.3 mM K^+, the concentration present in standard solution, and at 2.3 mM K^+, ectoderm cells from the gastrula, induced by lithium treatment of 2.5–3 hrs, differentiated as observed in the past into nerve and pigment cells. When, however, the lithium-induced cells were cultured in standard solution in which the K^+ concentration had been raised to 3.3–5.3 mM, networks of vacuolated cells were formed, which fused to form notochord-like masses. Optimal concentration of K^+ for producing this shift in differentiation is 5.3 mM. At 7.3 and 9.3 mM K^+, the cells differentiated into nerve and pigment cells, just as in 1.3 and 2.3 mM K^+. There is a gradual shift from pigment cells with few notochord cells at 3.3 mM K^+ to approximately 98% notochord with sparse pigment cells at 5.3 mM K^+.

From the experiments summarized in Table I it could not be determined whether K^+ exerted its transforming effect immediately after the initial lithium induction period, which we know to be Na^+-dependent and reversible (Barth, 1966), or whether the potassium ion acted during the days-long period preceding visible differentiation of induced cells. This question was resolved by the following type of experiment. Gastrula cell aggregates prepared as usual from explants of the ventral ectoderm were treated for 3 hrs with 71 mM Li^+ added to standard solution. All of the aggregates were transferred for post-treatment to standard solution in which K^+ was raised to 5.3 mM. After 4 hrs half the aggregates were transferred to culture dishes of standard solution containing 1.3 mM K^+. After 18 hrs of post-treatment in high K^+ the remaining aggregates were cultured in standard solution containing 1.3 mM K^+. The former group formed radial and ganglionic nerve and pigment cells with no networks or notochord. The 18-hr post-treated group formed networks of pre-notochord as well as extensive notochord, just as though they had been cultured continuously at 5.3 mM K^+. Thus it may be concluded that K^+ has its effect during the post-induction period and that the high level of potassium is required for more than 4 hrs but possibly less than 18 hrs if differentiation is to be shifted into the pathway that leads to notochord.

The significance of these observations on the effects of K^+ added to the medium at concentrations from 1.3–9.3 mM resides in their demonstration that the post-induction period of 4–18 hrs following the relatively brief initial "induction" treatment is a period during which shifts in ion ratios can play a decisive role in determining the path of differentiation a cell will take—whether toward ganglionic nerve, melanocyte, notochord, mesenchyme, *etc.* This post-induction period therefore becomes a focal point for further manipulation of ion ratios in an attempt to open up still other pathways of differentiation to the "induced" or "activated" cells.

Although K^+ is the key factor in completing the initial induction by lithium in these experiments, it was found that Ca^{2+} can modify differentiation of induced cells. As reported in Barth and Barth (1974) the differentiation of pigment cells is dependent upon the normal amount of Ca^{2+} in standard solution. Experiments cited in the 1974 paper proved that calcium can be lowered from the standard concentration of 0.74 mM to 0.2 mM in complete absence of Mg^{2+} for up to 18 hrs following lithium induction, and nerve and pigment cells differentiate in standard solution following such drastic post-induction treatment. On the other hand, if lithium-induced cells were treated *continuously* with the same low concentration of divalent cation (0.2 mM Ca^{2+} and 0 Mg^{2+}), pigment cell differentiation was inhibited. The amount of sodium in the culture medium was the standard concentration of 90.8 mM (2.8 mM from buffers). K^+ was present at 3.3 mM, which leads to notochord differentiation at standard concentrations of 0.74 mM Ca^{2+} and 0.83 mM Mg^{2+}.

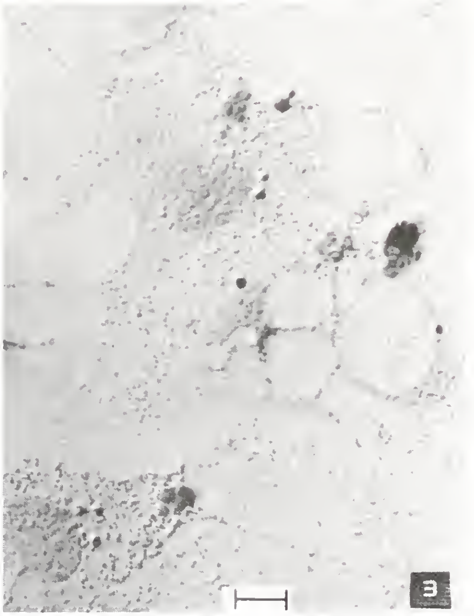
Inhibition of pigment cell differentiation is caused specifically by the 0.2 mM Ca^{2+} and 0 Mg^{2+} medium. Cells thus arrested in their differentiation contain a sharp, dense ring of slate-gray granules which persist without further change for as long as the cultures remain alive (up to 2–3 weeks).

Some preliminary experiments in which th Na/K ratios have been more drastically altered should be mentioned here. It is remarkable that the Na^+ concentration of the culture medium following lithium induction may be drastically lowered (from 90.8 mM to 41.8 mM), with simultaneous increase in K^+ (from 1.3 mM to 39 mM), and the aggregates survive and differentiate for at least a week. Lithium-induced cells cultured in standard solution form nerve and pigment cells, whereas lithium-induced cells cultured in the 1 : 1 ratio of Na^+ to K^+ form no nerve and the pigment ring cells are atypical and are probably pre-notochord cells. The ratio of Na^+ to K^+ in standard solution is 90.8 : 1.3.

Differentiation of notochord cells in cultures of gastrula ventral ectoderm

In standard solution, which contains 1.3 mM K^+, lithium-induced cells form sharp, clear pigment rings (Barth and Barth, 1959, Fig. 7) and differentiate into melanocytes. The first stages of this type of differentiation are apparent by the third or fourth day of culture, when the aggregates have become massive sheets of about 100 contiguous large clls. Within the next day or two most of these cells show an accumulation of pigment granules in the form of a ring near the nucleus in the cytoplasm. From this point on, beginning first in cells at the edges of the large sheets of "ring cells," there occurs a progressive transformation into dendritic cells with pigment rings dispersed to give uniform pigmentation. These large cells with amoeboid processes were identified as melanocytes (Barth and Barth, 1959).

When lithium-induced cells are cultured in standard solution with K^+ raised to 3.3, 4.3 or 5.3 mM, again pigment rings form; but the rings are from the beginning faint and diffuse. The following description of the subsequent behavior of this kind of pigment ring cell is based upon cultures in which K^+ in standard solution was raised to 5.3 mM, since this concentration gave the highest percentage of the typical high potassium effect. Frequently more than 95% of the cell aggregates followed this pattern of differentiation.



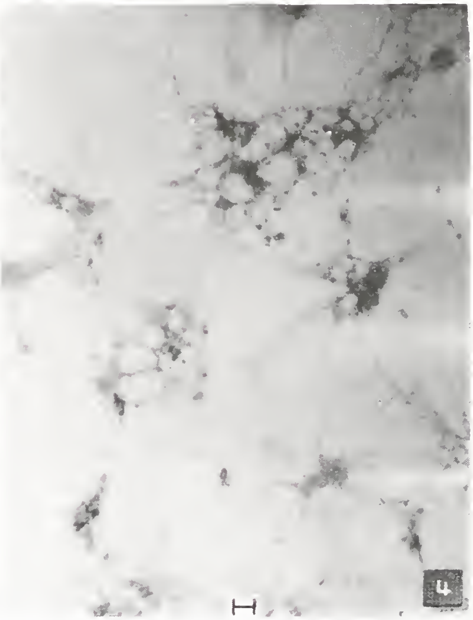


FIGURE 1. Cell aggregates treated with 71 mM Li^+ in standard solution for 3 hrs, were cultured in standard solution with high potassium (5.3 mM). Eight-day culture shows networks of cells containing small vaculoes. Scale bar represents 20 microns.

FIGURE 2. Same slide as Figure 1. Scale bar represents 20 microns.

FIGURE 3. Cell aggregates treated with 71 mM Li^+ for 4 hrs, were cultured in standard solution with 5.3 mM K^+. Nine-day culture shows fusion of vacuoles to form large vacuoles, interference contrast. Scale bar represents 20 microns.

FIGURE 4. Li^+ treatment for 4 hrs; fixed on day 11 of culture in standard solution with 5.3 mM K^+; fusions among cells with large vacuoles. Scale bar represents 20 microns.

The diffuse pigment rings disperse by about the sixth day of culture; cells become dendritic and the cytoplasmic extensions of individual cells make contact with adjacent cells. The result is an extensive network of cells. After about 8 days in culture the cells composing the network become filled with clear vacuoles, usually 10–12 or more per cell. Figure 1 is a low power view of this stage of early vacuolization within the network. A day or two later most of the cell bodies comprising the network are filled with vacuoles. Figure 2 shows at higher magnification two vacuolated cells fusing, and also shows cytoplasmic extensions to surrounding cells within the network which have not yet become vacuolated.

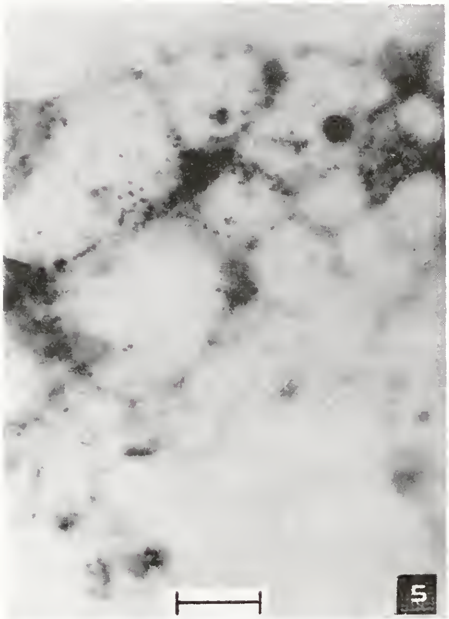
Within the next 24–48 hrs the small vacuoles coalesce so that the average cell now contains 4–6 large granules (Fig. 3). Continued fusions among vacuolated cells give rise to small masses, which become detached from surrounding masses. Figure 4 shows several vacuolated cells fused to give one small mass, as well as several nearby vacuolated cells in the process of joining the group. Figure 5, shows these vacuolated cells at higher magnification.

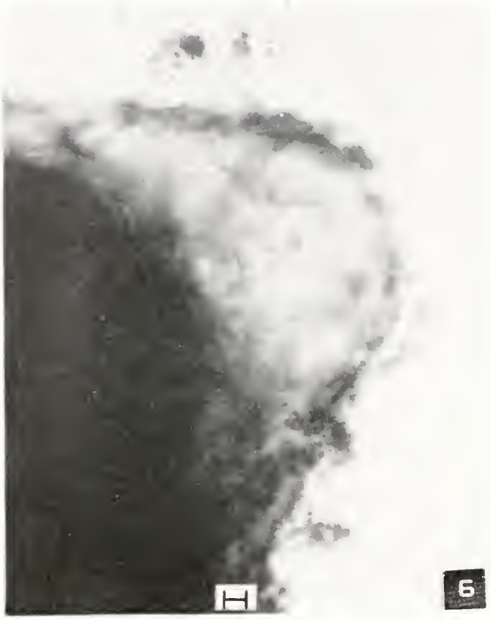
These small masses of cells filled with large vacuoles were highly suggestive of notochord. However, the vacuolated cells were smaller than those found in notochord differentiating *in situ* in the neurula, and the masses formed by spontaneous fusions among adjacent vacuolated cells were themselves too small to be identified with confidence as notochord.

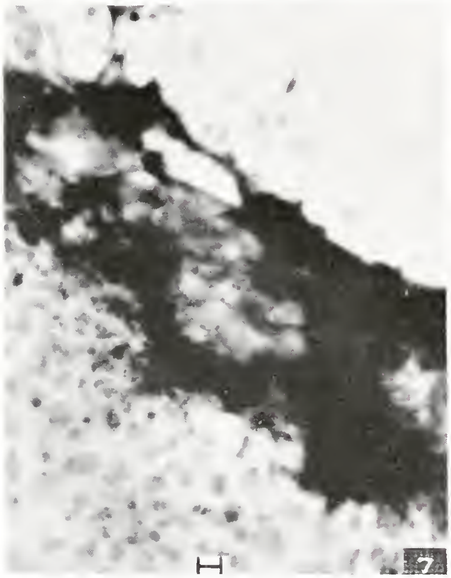
The next step was to dissect out notochord from the archenteron roof of stage 14 (early neural fold) embryos and stage 16 (neural tube). A center strip was excised that included not only prospective notochord but also the tightly adhering neural layers above. This larger explant was treated with EDTA briefly in order to loosen the pigment coat layer and the nervous layers. Dissection of small aggregates of cells from the notochord region then was possible. The samples contained also some nerve tissue and some lateral mesoderm from the archenteron roof. Cultured in standard solution containing 5.3 mM K^+, these aggregates differentiated to give some nerve and twitching muscle, but also formed masses of notochord with large vacuole-filled cells (Fig. 6). In some instances, individual vacuolated cells migrated away from the central mass and formed networks (Fig. 8). The similarity is striking between these networks of vacuolated cells obtained by dissection of normal embryos and the networks observed in lithium-induced gastrular ectoderm (compare Figs. 4 and 8).

An obstacle in positive identification of lithium-induced notochord cells was the small size of the masses formed by spontaneous fusions of vacuolated cells in the relatively thin population of cells migrating outward from an attached aggregate consisting of approximately 125 ectoderm cells per aggregate. In order to achieve a more normal cell mass and morphology, composites of 10–15 aggregates were prepared. This was done simply by swirling the dish to concentrate the aggregates after 2 hrs in the lithium treatment dish and pushing aggregates together by means of hair loops. Left for an additional hour in the lithium solution, the composites were sufficiently cohesive to be passed through a wash of standard solution and transferred to coverglasses in culture dishes containing standard solution with K^+ raised to 5.3 mM.

Figure 7, is an example of the differentiation observed in a composite of 10 fused aggregates on day 10 of culture. Within the main mass of cells one observes an inner curved, elongate group of vacuolated cells. The resemblance to notochord of this group of cells was unmistakable under the microscope.







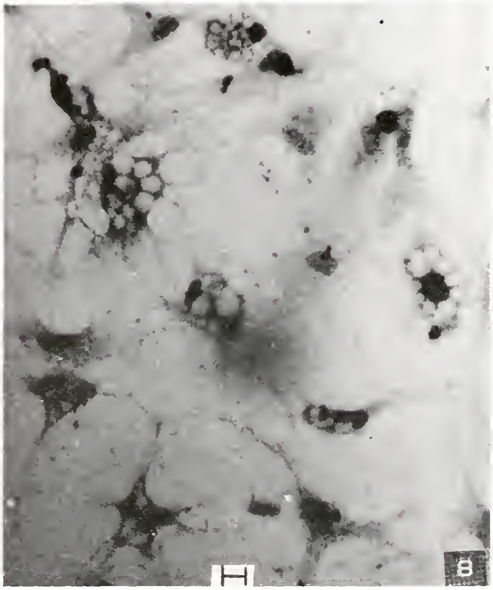


FIGURE 5. Same as Figure 4, showing large vacuoles within Li^+-treated aggregates cultured in high K^+ medium. Scale bar represents 20 microns.

FIGURE 6. Group of notochord cells derived from cells taken from archenteron roof at early neural fold stage; seven-day culture. Scale bar represents 20 microns.

FIGURE 7. Elongate mass of clear notochord cells within a mass of ectoderm cells produced by fusion of 10 cell aggregates. Before fusion, cell aggregates were treated with 71 mM Li^+ in standard solution for 3 hrs. The composites of 10 lithium-treated aggregates were cultured in standard solution with 5.3 mM K^+ for 10 days. Scale bar represents 20 microns.

DISCUSSION

The transformation of gastrula ectoderm cells into notochord by means of external K^+ extends the range of types of cellular differentiation already demonstrated to be subject to regulation by cations (Barth and Barth, 1969). In previous investigations various patterns of nerve and of neural crest derivatives have been obtained in a stepwise process in which the initial "induction" period is followed by a period of 4–18 hrs during which induction is reversible and depends upon the concentration of sodium in the medium. It is during this second period that a 5-fold increase in external K^+ causes later differentiation of notochord. While potassium at concentrations which can be applied continuously without killing the cells is not effective as an inductor, the same concentrations are very effective in modifying cells already induced by lithium. While Li^+ induces nerve and pigment cells in standard concentration (1.3 mM K^+ in standard solution), when K^+ is raised to 5.3 mM, notochord is induced but pigment cells are rare to absent (Table I). The third and final period preceding visible differentiation, although independent of K^+, is dependent upon the external concentrations of Ca^{2+} and Mg^{2+} (Barth and Barth, 1974).

Many years ago (1938) Holtfreter explanted small pieces of presumptive notochord from urodele and anuran gastrulae and found that they formed nerve and epidermis as well as notochord. It is possible that Holtfreter's solution lacks the required concentration of K^+ and that contact with the ectoderm that normally supplies the K^+ is necessary for notochord differentiation.

In a separate publication (Barth and Barth, 1974) we present a general theory with the objective of relating the considerable information thus far obtained from cell cultures (Barth and Barth, 1959, 1962, 1963, 1964, 1969) and from studies of whole embryos (Morrill, Kostellow and Murhpy, 1971; Kostellow and Morrill, 1968; Stableford, 1967) to ion regulation of normal embryonic induction. The general theory postulates a normal mobilization of cations from internal compartments. These cations would be trapped within the roof of the archenteron and the presumptive neural plate by the relatively impermeable outer surfaces of cells carried in during gastrulation. Concentration of cations then could result in local internal amounts and ratios of ions similar to those found to induce nerve, pigment cells and notochord when applied to small aggregates of gastrula ectoderm cells in culture.

The mechanisms whereby cations regulate cellular differentiation still are unknown. Numerous studies have provided evidence for a probable link between K^+ and regulation of protein synthesis in general, although the mechanism is not yet agreed upon. For example, Tupper (1973) in experiments on the sea urchin egg finds that increased K^+ exchange and protein synthesis closely parallel each other. He suggests that increases in K^+ permeability and K^+ decompartmentalization normally occur at fertilization and are reflected in change in membrane potential as well as in Na/K ratios. McDonald, Sachs, Orr and Ebert (1972), using external K^+ similar to those of the experiments reported here, attribute the effect

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FIGURE 8. Small masses of archenteron roof cells removed at neural tube stage were cultured in 5.3 mM K^+ in standard solution for 9 days. In addition to coherent masses of notochord cells as in Figure 6, other notochord cells migrated away from central masses and formed networks of vacuolated cells. Compare with Figure 4, which shows similar networks of vacuolated cells in cultures of lithium-induced gastrula ectoderm cells. Scale bar represents 20 microns.

of K^+ on DNA synthesis and growth to changes in membrane potential. Lubin (1967) reported that in mammalian cells, when amphotericin was used to cause loss of cell K^+, there was a parallel depression in rates of protein and DNA synthesis, reversible by adding K^+ to the medium. R. Steinhardt (personal communication to Dr. J. D. Ebert) suggests that the controlling factor in the changes in protein and DNA synthesis brought about by membrane depolarization by means of K^+ may be intracellular Ca^{2+} activity. Depolarization may result in increased permeability to Ca^{2+} as well as an inhibition of the Ca^{2+} pump. Renewed interest in ion changes at fertilization (Mazia, 1934; Heilbrunn, 1943) and early cleavage has involved application of electrophysiological methods to events at fertilization and early cleavage (Monroy, 1965; Epel, 1972; Weissenseel and Jaffe, 1972; Steinhardt, Shen and Mazia, 1972; Morrill *et al.,* 1971). Extension of similar methods to the gastrula stage should provide important clues to link changes in membrane potential to the kinds of changes in cation concentrations we now know are able to regulate cellular differentiation.

Studies on the regulation by ions of cell differentiation as distinct from general synthetic activity are still relatively meager. Lash, Rosene, Minor, Daniel and Kosher (1973) report an increase in cartilage formation and chondroitin sulfate synthesis in chick somites *in vitro* when the K^+ of the medium is increased from 2.69 mM to 4.68 mM. The stimulation persists even after the cells are returned to 2.69 mM K^+. This K^+ effect resembles ours in that the induction of notochord is obtained by changing the K^+ from 1.3 mM to 5.3 mM. Also the cells may be returned to 1.3 mM K^+ after 18 hrs and notochord differentiation will occur. Evidently some irreversible change occurs during 24 hrs exposure in Lash's system and during 18 hrs in ours.

Ionic regulation of chromosome activity in insect larval salivary glands is well known (Kroeger, 1963; Lezzi, 1970). The work of Kroeger and of Lezzi, in fact, provided impetus for the present experiments, since the high potassium effect reported here was an unexpected offshoot of an ongoing investigation of various Na/K ratios as related to differentiation of gastrula ectoderm cells. Beritashvili, Kvavilashvili and Kafiani (1969) have proposed that in the fish embryo the increase in K/Na ratio may play a part in switching over nucleic acid synthesis from replication to transcription paths.

Independent of mechanism, the experiments reported in the present paper provide definite proof that K^+ can switch differentiation from ectoderm to notochord, and further suggest that the potassium effect will most profitably be studied during the period following the initial induction. Whether the effect of K^+ is a direct effect of potassium concentration upon synthesis of new kinds of proteins or whether potassium acts indirectly through alteration of ATP utlization, through altering the Na/K ratio or still other mechanisms is unknown.

A final word as to the differentiation of K^+-induced notochord in cell cultures: The relatively small size of the cells and of the masses formed by fusion of these vacuolated cells observed in the present studies agrees with observations reported by Mookerjee, Deuchar and Waddington (1953). These workers isolated urodele notochord cells from gastrula and neurula stages by treatment with alkaline solutions at or above pH 7.6. They observed that isolated cells proceeded to become vacuolated but did not "attain as large a size as they would do within the embryo" (page 406). As reported in the present paper, larger masses of K^+-induced notochord cells could be prepared by fusing lithium-treated cell aggregates and culturing the composites in 5.3 mM K^+. The result was readily identifiable as notochord.

After the present work had been completed, the recent work of Takaya (1973) came to our attention. This investigator reports the formation of small numbers of *transient* vacuoles in differentiating cells of the newt embryo—ectoderm, muscle, cartilage, liver, *etc*. Only in notochord cells, however, Takaya notes that vacuole formation continues and vacuoles persist, with fusion of small vacuoles to form the larger vacuoles which characterize the differentiated notochord. Takaya's work on vacuolation during notochord formation in the embryo thus is in good agreement with the findings presented in the present paper concerning the induction of notochord from presumptive epidermis by means of the K^+ ion.

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We are indebted to Dr. James D. Ebert, Director of the Marine Biological Laboratory, for his encouraging interest in the research and for extending our knowledge about related investigations in progress in other laboratories.

Dr. Allan D. Dingle provided the photographs, as well as stimulating and critical comments, for which we express warm gratitude. To Miss Martha Coneybear we owe thanks for faithful and reliable technical assistance.

SUMMARY

Small aggregates of cells prepared from explants of ventral ectoderm of *Rana pipiens* gastrula were treated for 3–4 hrs in 71 mM Li^+ added to standard solution. When cultured in standard solution containing 1.3 mM K^+, the aggregates of ventral ectoderm cells differentiated into nerve and pigment cells. As the K^+ concentration in the culture medium was raised through 2.3, 3.3 and 5.3 mM, the frequency of pigment cells declined and networks of vacuolated cells were formed, which later fused to form small notochordal masses. Raising the K^+ to 7.3 and 9.3 mM gave nerve and pigment cells again, as in the lower potassium concentrations. The effect of high K^+ on lithium-induced cells becomes irreversible during the first 18 hrs. Cells may be returned to 1.3 mM K^+ after 18 hrs and notochord differentiation will occur.

Induced notochord cells first appear as somewhat larger cells which become filled with very small vacuoles by 7–8 days of culture. These small vacuoles then coalesce to form larger and larger vacuoles (9–12 days). The vacuolated cells aggregate into small clumps. Cultures were fixed and stained at various intervals for photography.

Cultures of small explants of notochord in 5.3 mM K^+ were observed to undergo the same type of differentiation. In both cases the notochord cells and vacuoles were smaller than in the whole embryo. Larger explants of notochord from archenteron roof gave large masses of notochord cells about the same size as in the whole embryo. When lithium-treated aggregates of the ventral ectoderm were fused together in 5.3 mM K^+ to form a composite of 10–15 aggregates, the notochordal masses were larger and the notochord cells were about the same size as obtained in comparable sized explants of notochord from the roof of the archenteron.

These findings are discussed in relation to the effects of high potassium on membrane depolarization, protein synthesis, DNA synthesis, and increase in intracellular Ca^{2+}.

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STROBILATION OF THE SEA NETTLE, *CHRYSAORA QUINQUECIRRHA*, UNDER FIELD CONDITIONS[1]

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The sea nettle, *Chrysaora quinquecirrha* (Desor, 1848), is locally abundant during summer along the east coast of the United States from southern New England to Florida. The venomous medusa stage of this species is a significant pest and a negative economic factor, particularly in the Chesapeake Bay region.

In common with many other cnidarians, *C. quinquecirrha* undergoes an alternation between polyp and medusa stages in its life history. While the medusa population dies off annually, the sessile polyp or scyphistoma stage may remain active all year and is potentially perennial (Truitt, 1939). The scyphistoma is capable of asexual reproduction, most commonly through podocyst formation. These cysts are also resistant to adverse environmental conditions. Given favorable conditions the scyphistomae undergo strobilation, a process leading to the production of free-swimming ephyrae. The proximal portion of the polyp remaining after strobilation undergoes renewed growth, returning the scyphistoma to normal size and morphology. It is then capable of continued asexual reproduction and perhaps repeated strobilation. The ephyrae develop rapidly into medusae, which are dioecious. Fertilization results in a free-swimming planula larva, which settles on a firm substrate and develops into the scyphistoma, thereby completing the life cycle.

While the morphological details of strobilation in *C. quinquecirrha* have been described (Littleford, 1939; Cones, 1969), little is known about the ecology and seasonal dynamics of the process. The only relevant field data available to date on strobilation in this species were based on collections of ephyrae by Cargo and Schultz (1966, 1967). The present study was undertaken to determine (1) when strobilation begins and ends in nature; (2) the percentage of polyps strobilating at a given time; (3) the number of ephyrae produced per strobila, as observed throughout the season; (4) whether a given polyp will strobilate more than once a season under field conditions.

METHODS AND HABITAT DESCRIPTION

Studies on strobilation in *C. quinquecirrha* were conducted from March 1972 through February 1973 in Sarah Creek on the York River, Virginia. Shells of the oyster, *Crassostrea virginica,* were collected using hand tongs from an old, abandoned shell accumulation in the Northeast Branch of the creek and returned to the laboratory in water-filled buckets. Examination of the shells was completed and the number of adhering scyphistomae and strobilae present determined within eight hours after collection. Samples were taken biweekly during March and from November through February. Weekly samples were collected during the first half of April and from August through October. From mid-April through July, sampling was conducted two to three times weekly and the results pooled for the entire week. Enough substrate was collected so that most samples contained in excess of 100 scyphistomae. Greater quantities of shell were necessary for winter and mid-summer samples because scyphistomae were less abundant during these seasons than in spring, late summer and autumn.

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In the laboratory, shells were immersed in a large preparation dish containing sea water and examined under low magnification (7–10 ×) using a dissecting microscope. As scyphistomae and strobilae were located and counted, they were dislodged from the shells and placed in Stender dishes for more detailed examination at higher magnification. Small polyps, as well as large ones, were counted during the study. Three methods were used in identification of scyphistomae: (1) examination of the nematocyst complement (Calder, 1971); (2) determination of mouth shape (Morales-Alamo and Haven, 1974); (3) identification of the ephyrae liberated following strobilation (Calder, 1972).

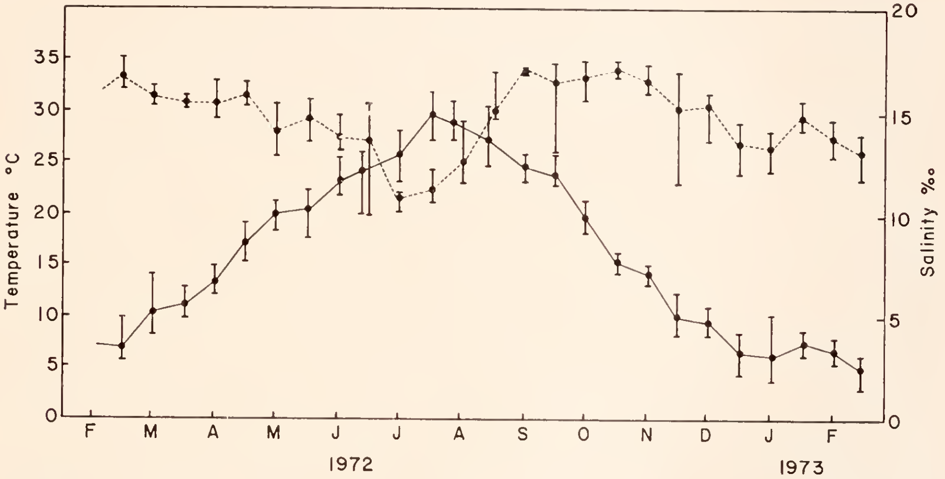

FIGURE 1. Salinity (dashed line) and water temperature (solid line) in Sarah Creek. Intersects indicate mean, vertical lines the range for the previous two-week interval.

All field-collected strobilae that had not liberated ephyrae prior to collection were placed individually in vials containing 4.5 ml of filtered sea water (17‰ salinity). These strobilae were held at 25° C (approximating ambient summer water temperatures) in an incubator until the total number of ephyrae produced could be determined. Identification was based on the morphology of the released ephyrae. After liberation of ephyrae, scyphistomae were removed to petri dishes and held in the laboratory until several had reattached. Upon reattachment, the exact location of each polyp was recorded and the petri dishes were secured to a retrievable rack and submerged, bottom up, in Sarah Creek. Scyphistomae were checked for restrobilation in the field several times weekly with a 10 × hand lens. New polyps produced by asexual reproduction during the study were removed from the dishes.

Hydrographic data, including salinity, dissolved oxygen, water temperature, and water transparency, were collected several times weekly in Sarah Creek throughout the study.

TABLE 1

Numbers of scyphistomae and strobilae in samples from Sarah Creek

Time interval	No. samples	No. scyphistomae	No. strobilae	Per cent of polyps strobilating
5 March–1 April	2	110	0	0.0
2–15 April	2	173	0	0.0
16–22 April	2	249	5	2.0
23–29 April	3	401	48	10.7
30 April–6 May	3	406	41	9.2
7–13 May	3	416	54	11.5
14–20 May	3	520	60	10.3
21–27 May	3	311	78	20.1
28 May–3 June	3	336	42	11.1
4–10 June	3	409	114	21.8
11–17 June	2	244	41	14.4
18–24 June	2	205	37	15.3
25 June–1 July	2	273	38	12.2
2–8 July	2	255	28	9.9
9–15 July	2	138	7	4.8
16–22 July	1	93	9	8.8
23 July–5 August	—	—	—	—
6–12 August	1	82	3	3.5
13–19 August	2	169	1	0.6
20–26 August	1	128	3	2.3
27 August–2 September	1	159	4	2.5
3–9 September	1	251	0	0.0
10–16 September	1	202	1	0.5
17–23 September	1	225	1	0.4
24–30 September	1	229	0	0.0
1–7 October	1	198	1	0.5
8–14 October	1	237	0	0.0
15–21 October	1	247	0	0.0
22–28 October	1	215	0	0.0
29 October–4 November	1	251	0	0.0
5 November 1972–3 March 1973	8	876	0	0.0

Sarah Creek is located on the north shore of the lower York River, 9 km from Chesapeake Bay. The creek divides into two branches, the Northwest and the Northeast, each having depths of 2 m for about 1.3 km (U. S. Coast Pilot, 1971). The shoreline is sandy at the entrance but elsewhere is predominantly muddy and bordered with saltwater cordgrass (*Spartina alterniflora*). The bottom consists of mud, except for accumulations of shell on private oyster grounds and near oyster houses. These shells provide substrate for a variety of epifaunal organisms, including scyphistomae of *Chrysaora quinquecirrha*.

Mean tidal range in the creek is about 0.9 m. Currents are weak except at the narrow entrance, where velocities reach about 0.5 m/sec. Hydrographic conditions fluctuate markedly in the creek (Fig. 1), both seasonally and in response to local weather conditions. Freshwater runoff from heavy rains rapidly decreases salinity and increases water turbidity. Salinities were below normal throughout the Chesapeake Bay system in 1972 due to heavy rains during the autumn of 1971. Unusually wet weather continued through the spring of 1972 and was followed by heavy rainfall during Tropical Storm AGNES in late June 1972 that further depressed salinities. In Sarah Creek salinities reached a minimum of 9.94‰ on 29 June. Waters of the creek are normally turbid, with maximum Secchi disc visibilities of about 2 m being attained in late autumn and early winter. In summer, visibilities are consistently below 1 m, and may drop as low as 0.3 m following heavy rainfall. Water temperatures increase or decrease rapidly depending upon air temperature. Although the creek occasionally freezes over in cold winter periods, it did not do so in 1972–1973. Summer water temperatures reached a maximum of 32° C. There was no evidence of oxygen depletion in creek waters during the study. Dissolved oxygen values averaged 10.1, 7.8, 7.1, and 8.4 mg/liter for winter, spring, summer and autumn, respectively. The lowest value recorded was 4.4 mg/liter following a heavy rain on 4 May 1972. The creek is sheltered from wind, and maximum wave height observed in the creek during the study was about 0.3 m.

TABLE II

Numbers of ephyrae liberated per strobila in samples from Sarah Creek. Strobilae having liberated ephyrae before collection were not included

Month	No. strobilae	No. ephyrae	Ephyrae/strobila
April	35	292	8.3
May	127	752	5.9
June	160	704	4.4
July	28	106	3.8
August	6	26	4.3
September	1	7	7.0
October	1	4	4.0
Totals	358	1891	5.3

RESULTS

Scyphistomae of *C. quinquecirrha* were collected in Sarah Creek throughout the year, although most survived as podocysts during the coldest part of the winter. Strobilation extended over a period of 24 weeks during the study. Strobilae were first observed on 18 April, when three of 115 polyps collected were beginning to show segmentation. Water temperature on this date was 16.8° C, having risen from 14.9° C four days earlier. After 20 April, when two additional strobilae were collected, numbers of strobilating polyps in the samples rapidly increased (Table I). Strobilation peaked during May and June, with a maximum weekly percentage recorded during 4–10 June. Peak value for a single sample was obtained on 5 June when 25.4% of the polyps collected were undergoing strobilation. Fresh water from Tropical Storm AGNES depressed salinities in Sarah Creek during late June (Fig. 1), but strobilation was already in decline and was not noticeably affected. Although strobilation continued throughout the summer, the percentage of polyps strobilating at a given time after June was relatively small. Only three strobilae were collected after August, the last on 2 October. These three were normal morphologically, and typical ephyrae were produced.

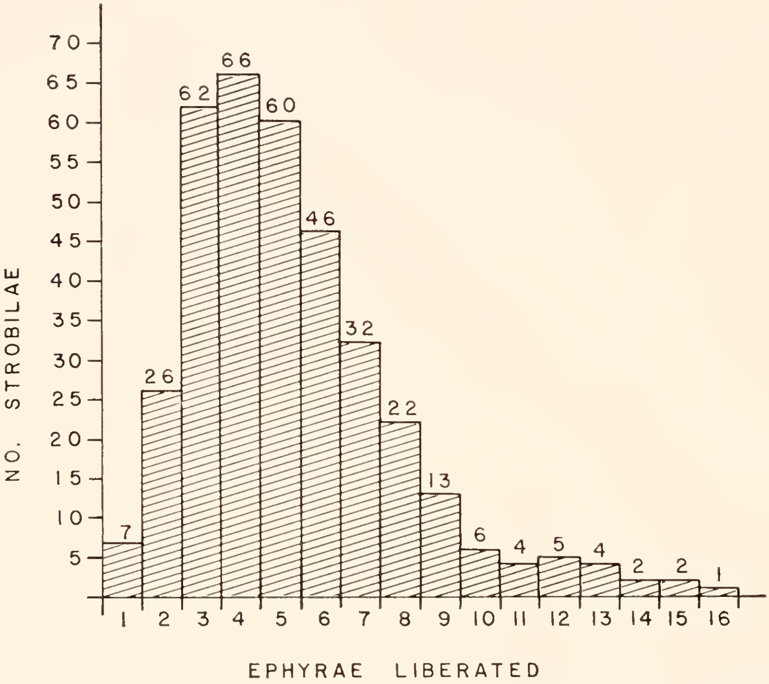

FIGURE 2. Frequency distribution of numbers of ephyrae produced per strobila.

Pulses of strobilation were evident from the samples. Peaks of strobilation for 23–29 April, 7–13 May, 21–27 May, 4–10 June, and 18–24 June were followed by lower levels on 30 April–6 May, 14–20 May, 28 May–3 June, 11–17 June, and 25 June–1 July respectively. These fluctuations did not correlate with any observed environmental factor except tidal rhythm. Strobilation peaks coincided with periods of increasing tidal amplitude as indicated in the 1972 tide tables (National Ocean Survey, 1971).

The number of ephyrae produced per strobila varied from one to 16 during the study (Fig. 2). The mean number of ephyrae per strobila was higher in spring than during the summer (Table II).

Studies conducted in late spring and early summer demonstrated that a scyphistoma can strobilate more than once in nature during a given season. Of 48 scyphistomae reimmersed in Sarah Creek following strobilation, 22 strobilated again once, 11 strobilated twice, four strobilated three times and two strobilated four times. Most of the scyphistomae were observed for a relatively brief interval before being lost; the mean length of time a polyp remained on the dishes was 24 days. The two polyps that restrobilated four times were monitored over an interval of six weeks. Losses of scyphistomae were attributed largely to nudibranch predation, although some evidently became detached during strobilation.

Discussion

Waters of the Chesapeake Bay system are characterized by a wide annual range in temperature. Under these conditions strobilation in *C. quinquecirrha* undergoes a definite seasonal periodicity. During this study strobilation began abruptly in April as the water temperature approached 17° C. After reaching a peak during May and June, the process did not terminate as suddenly as it had begun but continued at a diminished rate throughout the rest of the summer and early autumn. No strobilation occurred from November through March, when cold water temperatures prevailed. The influence of water temperature on strobilation in *C. quinquecirrha* has been determined experimentally in laboratory studies by Cones (1969) and Loeb (1972). In these experiments strobilation was induced by elevating the water temperature in cultures of polyps maintained under cold conditions. Manipulation of water temperature has also been used to trigger strobilation in other species of scyphozoans under laboratory conditions (Russell, 1970).

Water temperature is not always as significant in the control of strobilation as it is for *C. quinquecirrha* in Chesapeake Bay. Thiel (1962) concluded that water temperature was not very important in regulating strobilation of *Aurelia aurita* in the Kieler Förde. He found that strobilation could occur throughout the year, and that there were two peaks, both coinciding with plankton blooms. His conclusion that food supply was the major factor controlling strobilation in the Kieler Förde was further confirmed through supplemental feeding experiments.

In addition to temperature and nutrition, a number of other factors are known to affect strobilation, including salinity, dissolved oxygen, light, chemicals, symbiotic zooxanthellae, and pH (Spangenberg, 1968; Russell, 1970). As with temperature, the relative significance of these factors varies from species to species and from location to location. Even within a given species, populations from different geographic areas may respond differently to the same factor. In *A. aurita*, strobilation occurs at much lower temperatures in European populations than in populations from Texas (Spangenberg, 1965; Russell, 1970). As Russell suggested, such differences are probably due to the existence of a number of races in this widespread species.

A series of pulses in the rate of strobilation was evident during this survey. An analogous series of peaks in the abundance of ephyrae in St. John Creek, Maryland, was noted by Cargo and Schultz (1967). They hypothesized that (1) the early peak was due to strobilation of polyps having survived through the winter, (2) a second and larger peak was due to the excystment of polyps from podocysts and their subsequent strobilation, and (3) later peaks were due to repeated strobilation in polyps. While not disagreeing with this hypothesis, the pulses of strobilation observed in Sarah Creek occurred in such a regular succession that another explanation is possible. In being about two weeks apart, the peaks coincided with periods of increasing tidal amplitude, suggesting a semilunar periodicity in strobilation rate. It is also possible that chemical stimuli contribute to the pulses of strobilation. Loeb (1974) indicated that a neck-inducing factor (NIF) apparently plays a significant role in triggering strobilation in *C. quinquecirrha.*

Information regarding the number of ephyrae produced during strobilation in *C. quinquecirrha* varies considerably. According to Littleford and Truitt (1937), Littleford (1939), Truitt (1939) and Cones (1969), the number is relatively constant, varying only from five to six. Cargo and Schultz (1966) observed a range from three to nine, with a mode of five. Under laboratory conditions numbers from one to 16 (Crawford and Webb, 1972) and from five to 20 (Loeb, 1972) have been reported. The number produced by strobilae collected in nature during this study varied from one to 16, with a mean of five and a mode of four. Numbers of ephyrae per strobila were evidently highest early in the season. Monodisc strobilae were rare and typically smaller than those undergoing polydisc strobilation.

The phenomenon of strobilation in scyphozoans consists of two separate processes, namely segmentation and metamorphosis (Thiel, 1938; Spangenberg, 1968). This results in the derivation of several new organisms from the single original entity. In the segmentation process there remains after strobilation a small portion of the scyphistoma that is capable of totally regenerating itself and repeating the process. The occurrence of repeated strobilation in a given scyphistoma has been reported in several species. Gohar and Eisawy (1961) observed strobilation twice in succession within three weeks in polyps of *Cassiopea andromeda.* In *Mastigias papua* strobilation occurred three times in seven weeks (Sugiura, 1963), and *Cephea cephea* strobilated three times within a three week interval (Sugiura, 1966). Thiel (1962) demonstrated that a scyphistoma of *A. aurita* in the Kieler Förde could strobilate up to four times a year. In *C. quinquecirrha,* Littleford (1939) and Truitt (1939) noted that individual polyps underwent strobilation each summer for four successive years. Subsequent laboratory experiments by Cargo and Schultz (1967) and Loeb (1972) indicated that scyphistomae of *C. quinquecirrha* could strobilate repeatedly. Studies conducted in Sarah Creek during 1972 confirmed that repeated strobilation can also occur in nature.

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SUMMARY

1. A 12-month study of strobilation in the sea nettle, *Chrysaora quinquecirrha,* was conducted under natural conditions in the field. Observations were made in a creek of the Chesapeake Bay system where scyphistomae of *C. quinquecirrha* were known to occur and where medusae are normally abundant each year.

2. Strobilation was seasonal in occurrence, beginning in April and lasting until early October.

3. Maximum rates of strobilation occurred during May and June. Instead of a single sustained peak during this period, strobilation was protracted into a series of pulses that coincided with periods of increasing tidal amplitude, suggesting a semilunar periodicity. Although strobilation continued into October, the percentage of polyps strobilating after June was relatively low.

4. The number of ephyrae produced per strobila varied from one to 16, with a mean of five and a mode of four. The mean number of ephyrae per strobila was higher in spring than during summer.

5. Observations on individual scyphistomae attached to petri dishes confirmed that repeated strobilation can occur in polyps of *C. quinquecirrha* in nature.

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INITIATION OF METAMORPHOSIS IN LABORATORY CULTURED SEA URCHINS

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Many benthic marine animals release their gametes, embryos, or larvae into the water column. The offspring subsequently enter the adult population through settlement and metamorphosis.

Sea urchins are one such animal. They have highly differentiated larvae that undergo a complex metamorphosis. The scant information on their metamorphosis has been reviewed by Hyman (1955). MacBride (1903) was the first to prepare a comprehensive description of metamorphosis. He used *Echinus esculentus* eggs that were fertilized in the laboratory. The larvae were fed on organisms collected along with the seawater in which they were raised. Earlier workers used field-collected larvae. Neither of these methods could have yielded consistently healthy and uniformly developing animals. With the development of a culture technique for sea urchins (Hinegardner, 1969), large and uniform populations of healthy animals became available for study.

These laboratory raised larvae can reach a state of competence to undergo metamorphosis at about 21 days of age (Fig. 1a). Complete metamorphosis from feeding larvae to feeding adult takes 5 or 6 days. This includes the development of adult internal organs as well as the formation of the adult mouth and anus. The change in external shape from larval to adult, however, takes less than an hour. Parts of this sequence are illustrated in Hinegardner (1969). The terms we will use to designate larval and adult structures are from Hyman (1955). The series of events is: (1) the tube feet protrude from the opening in the vestibule. (2) The left postoral and left posterodorsal arms bend to the left of the anteroposterior axis. All the other arms bend to the right. This movement makes it possible for the urchin rudiment to reach the substratum (Fig. 1b). (3) The five primary tube feet attach to the surface and the spines protrude from the vestibule. (4) The thickened ciliary bands, called epaulettes, collapse and the tissue on the larval arms retracts from the spicules supporting them (Fig. 1c). The three pedicellariae come to lie evenly distributed on the aboral surface which has assumed a smooth rounded shape (Fig. 1d). These changes take an hour or less (Bury, 1895; Hinegardner, 1969).

After 24 hours the genital and ocular plates become more calcified. The spines, formed during the development of the urchin rudiment, elongate. The spines on the plates bearing the pedicellariae elongate also. At this point the exterior of the organism looks very much like a small adult urchin.

Very little is known about the factors that induce echinoderm larvae to settle. The larvae of the sea star, *Mediaster aequalis*, settle on the tubes of the polychaete, *Phyllochaetopterus prolifica*. If the tubes are absent, the larvae can delay metamorphosis up to 14 months (Birkeland, Chia and Strathmann, 1971). Sea

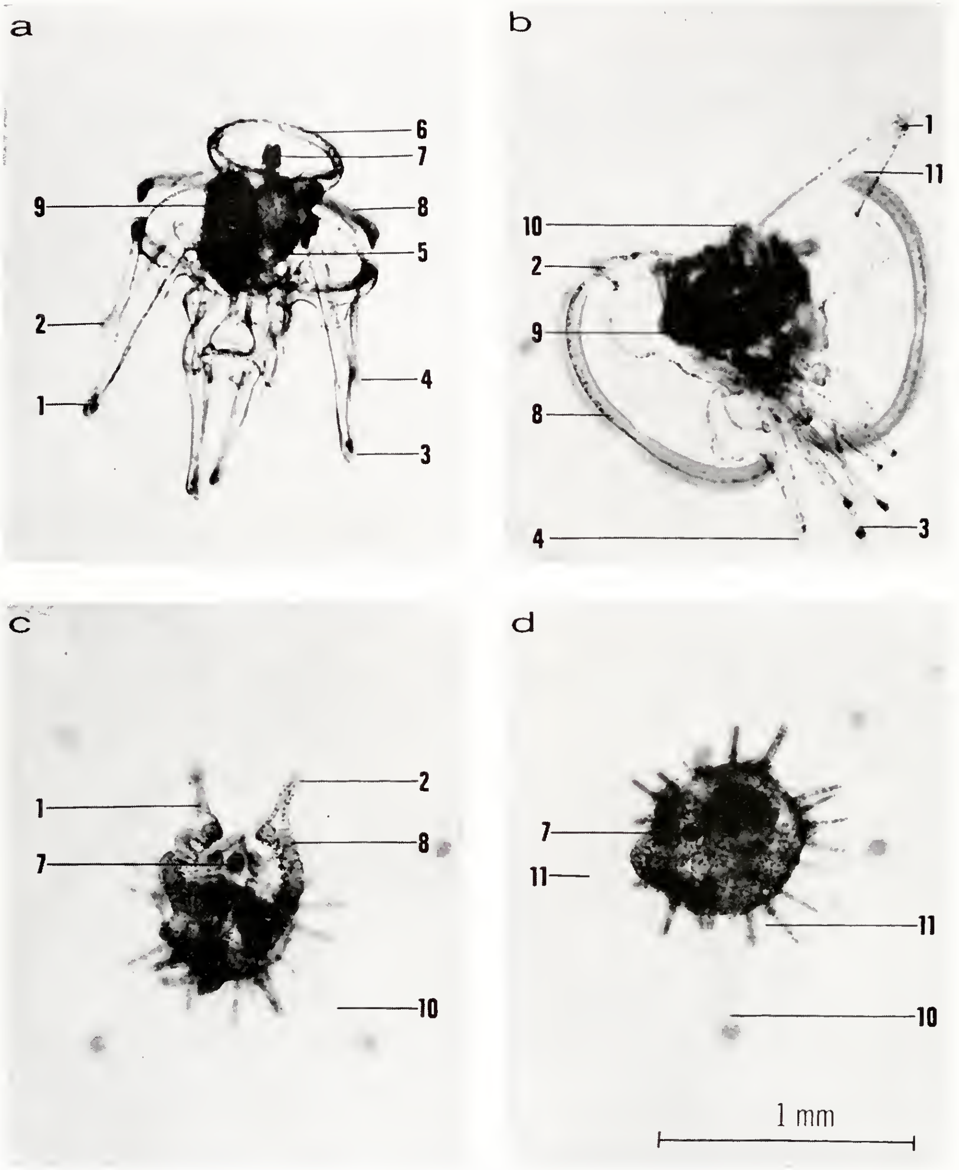

FIGURE 1. Metamorphosis of *Lytechinus pictus* (A.) A twenty-one day old larva competent to metamorphose is shown in frontal, or ventral, view with urchin rudiment on the left. (B.) A larva with arms bent, photographed 5 min after being placed in active seawater. This view is from below with the left side approaching the surface. (C.) This is an individual with much of the larval epithelium collapsed, 35 min after bending began, viewed from above, showing the folded ciliary band. (D.) An individual, viewed from above, in which the tissue collapse is completed, 60 min after bending began. One of the pedicellariae can be seen on the aboral surface. Legend is: 1, left postoral arm; 2, left posterodorsal arm; 3, right postoral arm; 4, right posterodorsal arm; 5, stomach; 6, upper epaulette; 7, apical pedicellaria; 8, lower epaulette; 9, urchin rudiment; 10 tube feet; 11, larval spicule.

urchins do not metamorphose spontaneously, either, but they will if presented with a film of bacteria and algae (Hinegardner, 1969). As we will show, only the bacteria are actually necessary.

MATERIALS AND METHODS

The stimuli that initiate metamorphosis in sea urchins were analyzed using the following procedure.

Larvae of *Lytechinus pictus* and *Arbacia punctulata* were reared from the gametes of adults maintained in the laboratory at Santa Cruz. During the summer of 1971, larvae of *Arbacia* were reared at Woods Hole, Massachusetts using local material. The larvae were reared by the method of Hinegardner (1969) with slight modifications. At the onset of feeding (48 hours post-fertilization), 400 healthy plutei were transferred to 6 l of 0.45 μm Millipore filtered seawater. The cultures were maintained at 18° C and stirred with a paddle attached to a 30 RPM clock motor. The cultures were fed thrice weekly and the water changed weekly. *Lytechinus* larvae were fed an unidentified species of the cryptophyte, *Rhodomonas*, and *Arbacia* larvae were fed the chlorophyte, *Dunaliella tertiolecta*.

Tests for the factors involved in the initiation of metamorphosis were performed in polystyrene petri dishes of 2 sizes: 60 × 15 mm and 35 × 10 mm. Sometimes glass petri dishes of the larger size were also used. In each experiment at least two controls were used. One was to insure the competence of the larvae to undergo metamorphosis. This was prepared by placing a plastic petri dish in an established aquarium for several days, whereby it acquired a bacterial film which is a potent stimulus for metamorphosis. The other control was for handling and ambient conditions. It consisted of sea water known not to initiate metamorphosis, either sea water from the larval cultures or fresh, filtered sea water. Ten larvae were added to each of the control and test dishes. The results were recorded after 4–6 hours and the dishes set aside to be checked for normal development after 24 hours.

When necessary, controls for both the possible inhibiting and enhancing effects of the materials used in chemical separations were included in the experiments. The material in question was added to a dish of sea water capable of initiating metamorphosis (*active sea water*). If no decrease in the number of larvae which underwent metamorphosis was observed, then the material was regarded as not inhibitory. Similarly, if no increase in metamorphosis was observed when the material was added to the sea water incapable of initiating metamorphosis (*inactive sea water*), then the material was regarded as not enhancing metamorphosis. Between the test and control dishes, a difference of 20% in the behavior of animals was regarded as insignificant.

The source of the sea water throughout these experiments was the Hopkins Marine Station, Pacific Grove, California. This water was collected and stored in opaque 60 l polyethylene barrels.

Quantities of crude active sea water were prepared in various polyethylene and glass containers of 4 to 20 l by incubating the particulate material from our aquarium filters, or the sediment from the bottom of the storage barrel, in sea water at 15° C for at least 2 days. Both pH and osmolarity were measured for each batch.

Initiation of metamorphosis can be induced electrically. We used standard neurophysiological apparatus, including a suction electrode with a tip diameter of 90 μm. The electrode was used in other experiments for just holding the larvae.

The role of surfaces in initiation of metamorphosis was tested using both glass and plastic petri dishes. The glass dishes were roughened with grinding compound and the plastic dishes with a small rotary brush. Glass spheres of 140 μm diameter were also added to a set of dishes.

RESULTS

The first three events of metamorphosis (extension of the tube feet, bending of the arms, and attachment of the tube feet) are reversible. The larva may bend its arms, then later return to the original larval shape. Sometimes a larva attaches its tube feet only to let go and swim away. The bending, but not the epaulette collapse and tissue retraction, can be induced by acid pH (6.5) or a slight decrease in osmolarity (from 1080 to 960 milliosmoles). The collapse and retraction are irreversible and, if they begin, metamorphosis follows.

TABLE I

Per cent metamorphosis of Lytechinus pictus *larvae on various combinations of inactive or active sea water, bacterial film and surface. See text for further explanation of experimental conditions.*

Sea water	Film	Surface	% Metamorphosis	No. tested
Inactive	Absent	Glass	0	10
	Absent	Glass spheres	0	10
	Absent	Rough glass	0	10
	Absent	Plastic	3	200
	Absent	Rough plastic	0	10
	Present	—	62	40
Active	Absent	Glass	97	30
	Absent	Glass spheres	70	10
	Absent	Rough glass	75	20
	Absent	Plastic	93	200
	Absent	Rough plastic	75	20
	Present	—	90	200

A crude system that induces complete metamorphosis in both *Lytechinus* and *Arbacia* larvae is a petri dish with a prepared bacterial film (see Materials and Methods). Tests of individual components of this system (bacterial film, surface of the dish and water) on *Lytechinus* larvae showed that while the film was important, the critical component was also in the water (Table I). Whether the surface was glass or plastic, rough, smooth or covered with glass beads made little difference. Neither adult *Lytechinus* from our aquaria nor the brown alga *Macrocystis* on which they fed provide the cue for metamorphosis when placed in freshly filtered sea water. The osmolarity of active sea water which provided the cue for metamorphosis of both *Lytechinus* and *Arbacia* larvae did not differ from that of filtered sea water. The pH was 7.4–7.6, while filtered sea water was

8.0–8.2. Raising the pH of active sea water to 8.2 did not alter its ability to induce metamorphosis.

The volatility of the active factor in sea water was tested by placing two containers beneath a 500 ml bell jar. One was a test petri dish with larvae in 10 ml of inactive sea water and the other contained 200 ml of active sea water. After 24 hours, the ability to initiate metamorphosis was not transferred through the air. Furthermore, the active factor was not driven out of the active sea water by converting it to an aerosol which was then collected.

The ability of decolorizing charcoal (Norit A, Amend Drug and Chemical Co., New York, New York) to remove the potential to initiate metamorphosis from active sea water was tested. The charcoal was added to filtered active sea water and then removed by filtration through Whatman #1 filter paper. During this treatment active sea water changed from a pale yellow to colorless and lost the ability to initiate metamorphosis; however, it was no more inhibitory when mixed with active sea water than was filtered sea water. Other possible effects of the charcoal were excluded by the appropriate controls.

The organic nature of the active factor was tested by ashing. Eight grams of particulate matter were removed from one liter of active sea water by filtration through Whatman #1 filter paper and then ashed in a porecelain crucible to constant weight over a gas flame in air. This yielded a gray powder. No metamorphosis occurred when the ash was added to filtered sea water in one, five and ten times the original concentration. Appropriate controls excluded any inhibition by the ash.

The activity will pass through a 0.22 μm Millipore filter, indicating that it is not carried on small particles. Sometimes the activity becomes trapped on the particulate matter retained on the filter. This can be avoided by pre-centrifugation for half an hour at 13,000 g. The Millipore filter must be rinsed before use with at least 10 ml of sea water or glass distilled water per square cm of surface in order to remove toxic components.

Activity will also pass through a centrifuge ultrafilter (Centriflo filter, Amicon Corp., Lexington, Massachusetts), which excludes molecules of 50,000 MW and larger. The filter cones were thoroughly washed with glass distilled water and filtered sea water before use.

The ability of the active factor to pass through a dialysis membrane was tested. Dialysis tubing (Van Waters and Rogers, San Francisco, California, pore size = 24 Å) was prepared by boiling 20 minutes in sodium dodecyl sulfate, boiling 10 minutes in glass-distilled water and soaking 20 minutes in 10^{-4} M EDTA, followed by storage in glass-distilled water. In another method of preparation, the tubing was merely soaked in sea water before use. We found no difference between these two methods. A twenty milliliter aliquot of active sea water was placed inside the tubing and 5 ml of inactive water outside. The fractions were tested after 4 hours. The active factor passed through the dialysis membrane. Controls showed that the membrane had no effect itself. The rate the activity moved through the membrane suggests that the active element had a molecular weight less than 5,000.

We have found that electrical stimulation of competent *Arbacia punctulata* larvae induces the events of metamorphosis through tissue retraction from the larval arms. A stimulus of 150 volts delivered for one millisecond at one second intervals induces bending of the arms and retraction of the tissue from them. In ten trials, the average time until the beginning of retraction from the posterodorsal arms was 223 seconds (s.d. = 148). Because the larval epithelium must be sucked into the bore of the pipette to hold the actively moving larva, the rest of the contraction would only occur if the larva was released. When it was, metamorphosis was normal and complete. *Lytechinus pictus* larvae did not undergo metamorphosis in response to electrical stimulation.

In about 80% of our tests, *Lytechinus* and *Arbacia* larvae would not undergo metamorphosis in active sea water if the rudiment tube feet were kept from touching a solid surface. This was done by holding the animals on a suction pipette or simply by turning them up side down and holding them that way. The remaining 20% did metamorphose without tube feet attachment. Repeated brief touching of the larval epithelium, vestibular pore, adult spines or even the tube feet would only cause bending of those that would not metamorphose. However, if the larvae were allowed to hold on to a solid surface for several minutes complete metamorphosis was induced.

Larvae will metamorphose in active sea water with various portions of the epithelium removed by surgery. Several individuals metamorphosed even though the entire upper epaulette was removed.

Discussion

We have identified two stimuli that are needed for the initiation of metamorphosis: (1) a chemical cue is of prime importance, (2) tube foot attachment to a surface is usually necessary. Experiments suggest that the chemical cue is a non-volatile compound of bacterial origin. It is probably organic since it is lost by ashing and removed by decolorizing charcoal. It is non-particulate since it passed through a 0.22 μm Millipore filter, a cellulose acetate ultrafilter, and a dialysis membrane. The characteristics of the ultrafilter put the molecular weight at less than 50,000. The speed of passage through the dialysis membrane suggests the molecular weight is probably less than 5,000.

Bacterial films and the sea water associated with bacterial and organic particulate matter kept in the dark, can both induce metamorphosis. This indicates that the factor is of bacterial origin. Bacterial films have been implicated in the settlement of other larvae (Wilson, 1955; Meadows and Williams, 1963). Large beds of the sea urchin *Strongylocentrotus purpuratus* associated with leptopel near sewage outfalls have been reported (North and Pearse, 1970). Perhaps these populations arose from larvae settling in regions rich in organic matter and bacteria. Formation of the factor appears to require special conditions. So far we have not been able to isolate a bacterium from the active sea water preparations that can initiate metamorphosis or that can impart that capacity to the sea water in which it was grown. Several enriched and defined media have been tried.

Efforts have been made to understand the stimuli for settlement of other marine animals (see review by Meadows and Campbell, 1972). For example, the serpulid polychaetes, *Spirorbis* spp., exhibit striking substrate preferences. Four different species of this genus each prefer a different species of alga for settlement (de Silva, 1962; Gee and Knight-Jones, 1962). The specific stimulus that

Spirorbis larvae receive from algae is chemical (Williams, 1964; Gee, 1965). The cyprid larvae of barnacles settle in response to an adsorbed chemical (Crisp and Meadows, 1963). Sediments are important in inducing the settlement of the larvae of the snail, *Nassarius obsoletus*. The inducing properties of these sediments can be transferred to the adjacent water (Scheltema, 1961). On the other hand, Wilson (1952) has shown that *Ophelia bicornis* larvae settle on sands taken from the area in which the adults live even if these sands are treated to remove organic matter. Also his experiments suggest that some sands carry a substance repellant to settlement. The work reported here suggests the presence of a specific attractant to metamorphosis in sea urchins.

We have not been able to extract and recover the chemicals necessary for the initiation of sea urchin metamorphosis using published methods (Jeffrey and Hood, 1958; Siegel and Degens, 1966). The active element is probably present in very low concentrations, as suggested by the fact that it is sometimes removed by particles caught on membrane filters. Another problem has been our inability to remove all toxic contaminants from organic chemicals used in extraction procedures. The acid pH of active sea water suggested the use of anion exchange resins as a method of separation. But even the eluents from extensively washed resins were toxic to the larvae.

The form assumed by a dying larva looks enough like a metamorphosed individual to be confused with it. Only by observing test animals after 24 hours can healthy young urchins be distinguished from dying larvae. None of the early workers did that and as a consequence erroneous conclusions have been drawn. For example, experimental work by Huxley (1928) using mild mercuric chloride poisoning, led him to suggest that metamorphosis was due to metabolic differences between larval and adult tissues. He was probably seeing dying animals.

How the larvae bend during metamorphosis is not well understood. Von Ubisch (1913) reports muscle bundles in the wall of the larval somatocoel. These bundles could provide the force to bend the larva. This is compatible with the reversible nature of the bending and its initiation by changes in pH and osmolarity.

The manner in which the larvae interact with the chemical cue is unknown. Since all portions of the larval epithelium have been surgically removed in one or another of the ablation experiments, it is safe to say that a discrete sensory structure does not exist there. Since the tube feet respond to a surface, possibly they or other parts of the urchin rudiment begin the response to the chemical cue. Another possibility is that the chemical interacts with the cells in the epithelium. Changes in surface properties could be allied with cell movement. Electrical stimulation could bring about such changes. Studies are being undertaken to address these questions. Further characterization of the chemical cue must await more sensitive and efficient organic chemical methods for sea water.

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SUMMARY

A method is described for studying the initiation of metamorphosis in laboratory cultured sea urchins. Two stimuli are usually needed to initiate metamorphosis: a non-particulate organic chemical cue and a surface. The chemical probably has a molecular weight of less than 5,000 and is of bacterial origin. Electrical stimulation can be used to initiate metamorphosis of *Arbacia punctulata* larvae.

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MORPHOLOGY, HEMATOLOGIC PARAMETERS, AND BEHAVIOR OF HEMOLYMPH CELLS OF THE QUAHAUG CLAM, *MERCENARIA MERCENARIA*[1]

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Molluscan hemolymph cells have been implicated in diverse functions, including an active role in internal defense against invading foreign materials, biotic and abiotic (see reviews by Feng, 1967; Cheng, 1967; Cheng and Rifkin, 1970). The immediate fate of soluble and particulate materials introduced into molluscs is well known. Specifically, injected particulate materials are phagocytosed (Stauber, 1950; Tripp, 1958a, 1958b, 1960, 1961; Feng, 1959, 1965a, 1965b, 1966a, 1966b; Arcadi, 1968; Cheng, Thakur and Rifkin, 1969; Pauley and Krassner, 1972) unless they are too large, in which case they are encapsulated (see reviews by Cheng, 1967; Cheng and Rifkin, 1970). It would thus appear imperative that the mechanisms involved in phagocytosis by molluscan cells be examined in detail if their role in internal defense is to be elucidated. Furthermore, it would be of interest to ascertain the ultimate fate of phagocytosed substances at the chemical level. However, before any detailed studies relative to these functions of molluscan leucocytes can be achieved, it is necessary that the numbers and types of hemolymph cells, as well as their normal behavior, be ascertained.

The purpose of this paper, therefore, is to describe the morphological and behavioral characteristics of the circulating cells of the hemolymph of *Mercenaria mercenaria*. Such descriptions are obviously essential as a preface to studies concerned with their function as related to internal defense. In addition, an attempt has been made to establish hematological parameters in this mollusc in order to determine their normal values and to ascertain whether these are interrelated. Samples of *M. mercenaria* collected from two different geographic areas were utilized to provide comparison.

MATERIALS AND METHODS

Fresh specimens of the quahaug clam, *M. mercenaria*, from Buzzard's Bay, Massachusetts, and Great Bay, Long Island, New York, were obtained from a commercial source and held in the laboratory for 1 week prior to use. They were maintained at 19–20° C in a recirculating seawater system at a salinity of 20–25‰.

A 1.5 ml sample of hemolymph was withdrawn with a hypodermic needle and syringe from the anterior adductor muscle sinus of each clam by the method of Feng, Feng, Burke, and Khairallah (1971). One-half ml of the sample was immediately discharged onto a clean glass slide while the other 1 ml of hemolymph was emptied into a 10 × 75 mm test tube containing 0.03 ml of 50% glutaraldehyde.

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TABLE I

Total cell count and packed cell volume of Mercenaria mercenaria *from two geographic locations* (*n* = 24 *from each group*)

		Buzzard's Bay	Great Bay
Total cell count (per mm^3)	$\bar{x}$ =	1954.9	1411.7
	sd =	1062.7	879.9
Packed cell volume	$\bar{x}$ =	0.46%	0.34%
	sd =	0.27%	0.14%

Each tube was immediately capped and the contents mixed by shaking. Packed cell volumes (percentage of hemolymph volume occupied by cells), total cell counts, differential analyses, and measurements of cell sizes were made using the cells from the latter preparation. Packed cell volume was measured by use of the microhematocrit method while total cell counts were obtained with a hemacytometer. Ten cells of each type from 24 clams from each location were measured. All cells, including those observed in stained smear preparations, were measured by use of a calibrated eyepiece micrometer.

Fixed and stained smears

The hemolymph placed on glass slides was permitted to settle for 10 minutes at room temperature (21° ± 1° C), after which the adhered cells were fixed by flooding the slides with 3% seawater-glutaraldehyde. Subsequently, the attached cells were rinsed in distilled water for 2 minutes, dehydrated in 95% ethanol for 1 minute, and air dried. The cells were then stained with 4% Giemsa's stain in Sorenson's buffer, pH 6.5, for 20 minutes, rinsed with fresh buffer followed by distilled water, and air dried.

Estimates of the ratio of nuclear area to total cell area in spread, fixed, and stained cells were obtained by the following method. The length of the cell or nucleus was multiplied by its width and this product for the nucleus was divided

TABLE II

Mean sizes of immediately fixed leucocytes from two groups of Mercenaria mercenaria *of 24 animals each* (*n* = 240 *for each cell type from each group*). *The two types of agranular cells are indistinguishable in immediately fixed preparations. All measurements are given in* μm.

		Great Bay		Buzzard's Bay	
		Length	Width	Length	Width
Granular cells	$\bar{x}$ =	12.10	10.36	13.49	11.73
	sd =	2.61	1.33	1.82	1.89
Agranular cells	$\bar{x}$ =	10.35	8.79	12.02	9.87
	sd =	1.33	1.07	1.43	1.27

TABLE III

Differential counts of immediately fixed and stained spread leucocytes of Mercenaria mercenaria *from two geographical areas. The two types of agranular cells are indistinguishable in immediately fixed preparations.*

	Buzzard's Bay	Great Bay
	Immediately fixed leucocytes	
Granulocytes	$\bar{x}$ = 58.40%	65.70%
	sd = 6.31%	7.87%
Agranular cells	$\bar{x}$ = 41.60%	34.30%
	sd = 6.31%	7.87%
	Stained spread leucocytes	
Granulocytes	$\bar{x}$ = 67.73%	67.61%
	sd = 13.64%	14.47%
Fibrocytes	$\bar{x}$ = 24.07%	19.60%
	sd = 14.33%	6.75%
Hyalinocytes	$\bar{x}$ = 8.27%	12.88%
	sd = 2.29%	14.35%

by the product for the entire spread cell. One hundred cells of each type from clams obtained from the two collection areas were sampled. It is recognized that the nuclear to total cell area ratios obtained by this method are only approximations since the spread cells and nuclei are of various shapes, but for the purpose of comparison are assumed to approach ellipsoids in their average dimensions. Based on this assumption, in ascertaining these ratios, that portion of the area of the rectangle tangential to the four sides not included in the ellipse was subtracted. It is noted that the subtracted area is constant irrespective of whether the actual shape of the included ellipsoid is a circle or a regular ellipse.

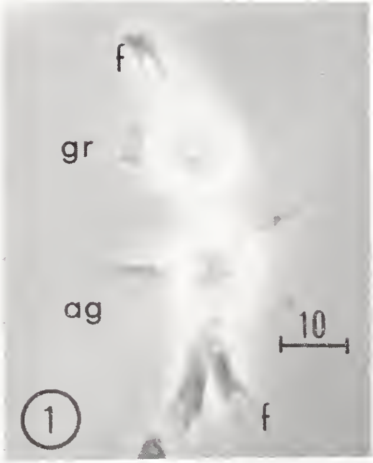
All data were processed on a CDC 6400 computer, utilizing a packaged statistics program, the Lehigh Amalgamated Package for Statistics (LEAPS). The coefficient of correlation used was Pearson's Product-Moment.

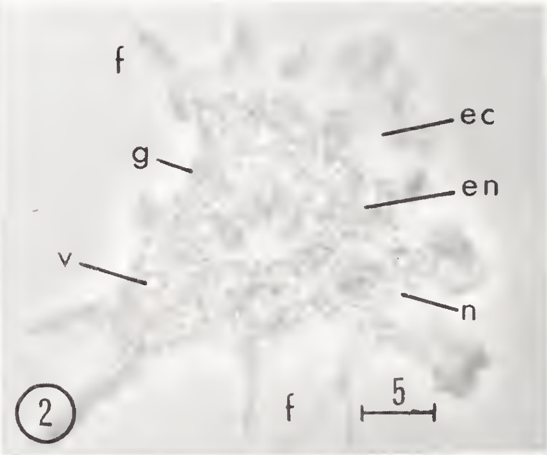
Fresh cells

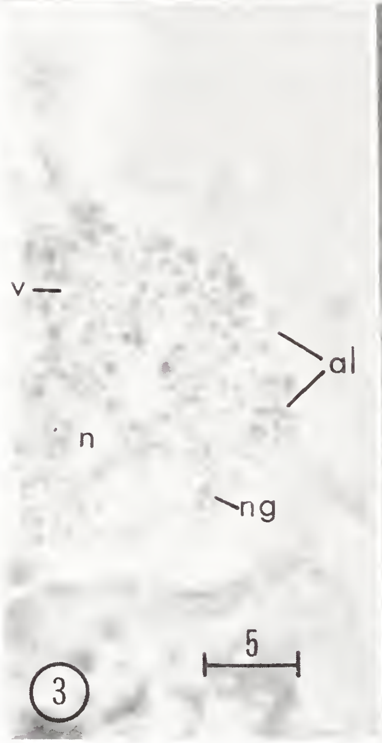
Fresh leucocytes obtained from the anterior adductor muscle sinus of several clams from both locations were observed in hanging drops or as sealed wet mounts using brightfield, phase, and Nomarski interference optics. All observations were made at room temperature (21° ± 1° C).

Vital staining

One drop of a 0.01% solution of Janus Green B or neutral red in seawater was added to four drops of fresh hemolymph on slides and a No. 1 coverglass, ringed with petroleum jelly, was gently applied. Cells were observed for up to 1 hour following exposure to the dyes and the uptake of stains by various organelles was observed.







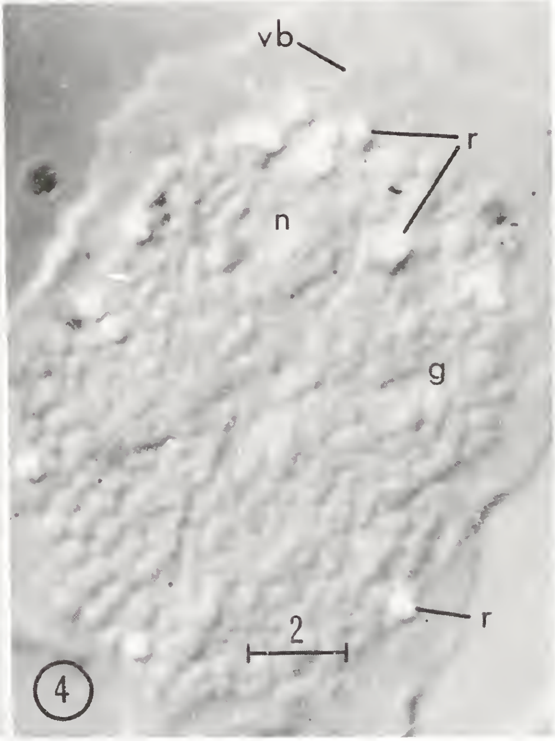


FIGURE 1. Figures 1–4 are living leucocytes of *Mercenaria mercenaria* with scale-bar units in μm; Figure 1, contracted granulocyte (gr) and agranular (ag) leucocytes, with filopodia-like projections (f) (phase contrast).

FIGURE 2. Spread granulocyte showing the separation of endoplasm (en) and ectoplasm (ec), filopodia-like projections (f), and various inclusions including granules (g), vacuoles (v), and nucleus (n) (phase contrast).

FIGURE 3. Spread granulocyte enclosing both normal (ng) and altered (al) granules (phase contrast).

RESULTS

The mean dimensions of the 48 clams from both groups were 8.98 ± 0.55 cm in length, 7.30 ± 0.40 cm in width, and 4.84 ± 0.29 cm in thickness. Based on these measurements, the clams from both groups fall into a small size range.

Total cell counts and packed cell volumes of hemolymph from clams from Buzzard's Bay and Great Bay are included in Table I.

Living leucocytes in vitro

Living leucocytes of *M. mercenaria*, when first placed on slides are mostly irregularly oval or spindle-shaped. Two general varieties of leucocytes, granular and agranular, are easily distinguished by their sizes and appearances in the living, contracted state (Fig. 1). Measurements of contracted leucocytes are given in Table II. Differential analyses of these cells are presented in Table III.

Structurally, both granular and agranular cells include one or more tufts of filopodia-like protrusions (Fig. 1). These tufts are approximately one-half the diameter of the cell in length and move in a three-dimensional sweeping motion.

Nuclei and cytoplasmic inclusions are visible in contracted granular cells but are difficult to define because of the refractiveness of the many cytoplasmic granules present (Fig. 1).

The cytoplasm of granular cells is hyaline and slightly refractile. Few cytoplasmic inclusions are visible except for a few refractile bodies situated near the large, oval nucleus.

Cell adherence and spreading

Upon contacting the slide, both granular and agranular leucocytes adhere and commence to spread. Contact between an attached and a non-attached cell usually results in adhesion of the two. Such cells may be of the same or different types. Occasionally, clumps of from two to several hundred adhered leucocytes can be seen immediately subsequent to slide preparation. Within 5–30 minutes at 21° ± 1° C, many of the cells from these clumps migrate outward over the substrate. It is noted that leucocytes will adhere to and spread on both upper and lower glass surfaces.

Spreading of individual cells begins with adherence to the slide. Cells that do not adhere do not spread.

Spreading of specific cell types

The spreading behavior of *M. mercenaria* leucocytes is of significance in cell identification. Specifically, three types of cells can be distinguished by this feature in conjunction with others. The cells referred to earlier as granular cells are being formally designated as granulocytes while those referred to as agranular cells can be identified as of two types being designated as fibrocytes and hyalinocytes. This

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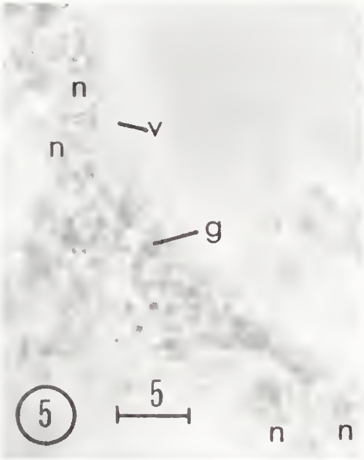
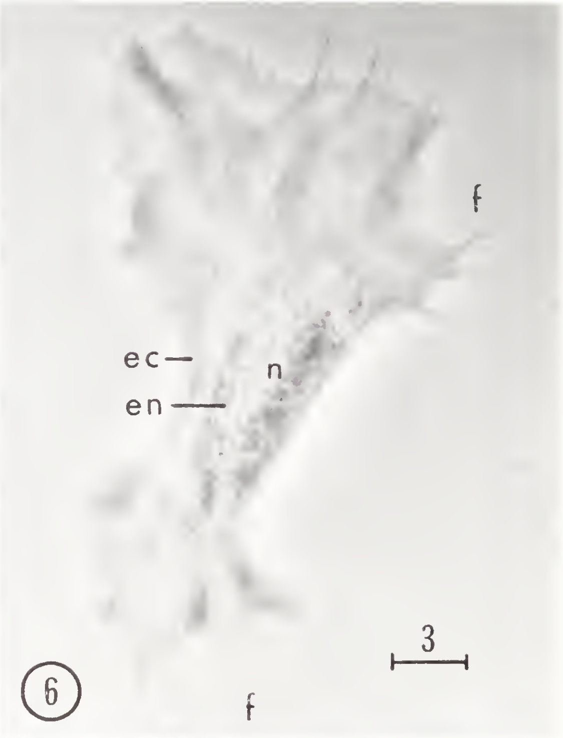
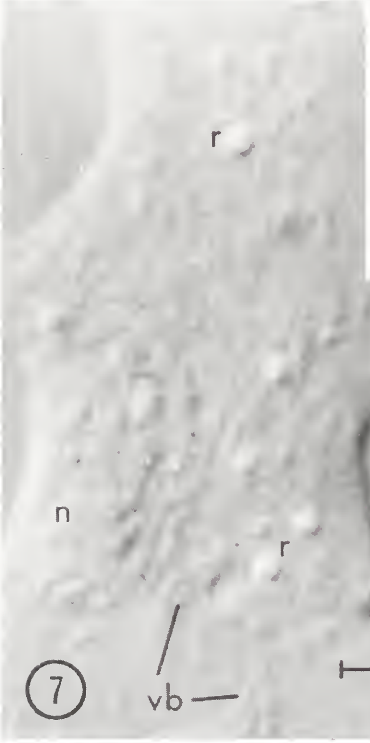
FIGURE 4. Spread granulocyte. Note vermiform body (vb) in the ectoplasm (ec); and the nucleus (n), refractile bodies (r), and granules (g) in the endoplasm (en) (Nomarski interference).







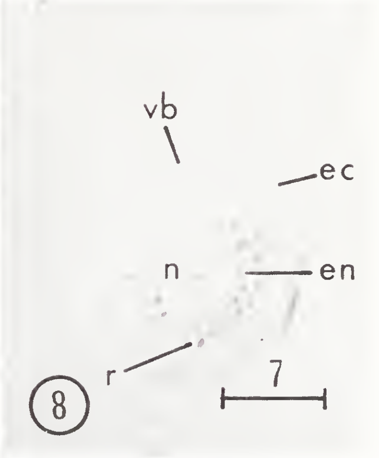


FIGURE 5. Figures 5–8 are living leucocytes of *Mercenaria mercenaria* with scale-bar units in μm; Figure 5, multinucleate, giant granulocyte containing four nuclei (n), granules (g), and vacuoles (v) (phase contrast).

FIGURE 6. Fibrocyte showing ectoplasm (ec) with filopodia-like projections (f), endoplasm (en), and acentric nucleus (n) (phase contrast).

FIGURE 7. Fibrocyte containing refractile bodies (r), nucleus (n), and vermiform bodies (vb) (Nomarski interference).

FIGURE 8. Hyalinocyte showing ectoplasm (ec), endoplasm (en), containing refractile bodies (r), and vermiform bodies (vb) (Nomarski interference).

terminology is being adopted after Foley and Cheng (1972), who have identified morphologically similar cells in the hemolymph of the American oyster, *Crassostrea virginica*. Descriptions of these three types of cells of *M. mercenaria* follow.

Granulocytes. Subsequent to adherence, the cytoplasm of each granulocyte flows out along the substrate in an irregular pattern. At the same time, cytoplasmic refractility decreases and the cytoplasmic inclusions become more visible (Figs. 2, 3). The endoplasm of the spread cell contains at least four types of identifiable inclusions other than the nucleus. These are: (1) vermiform bodies (possibly mitochondria), which are thin and elongate, reaching lengths of about 2 μm (Fig. 4); (2) vacuoles from less than 1 to 4 or 5 μm in diameter and which may contain inclusions (Figs. 2, 3, 5); (3) small, spherical refractile inclusions each averaging 0.7 μm in diameter and which are most readily recognizable when observed with Nomarski interference optics (Fig. 4); and (4) granules, which are the most conspicuous of the inclusions in the granulocytes (Figs. 2–5). Each of these granules averages 0.8 μm in greatest diameter. They are alterable in shape and may vary from being spherical to elongate (Fig. 3).

Surrounding the endoplasm is the hyaline, agranular ectoplasm, the periphery of which is undulating. Beyond this border extend a variable number of spike-like projections, each measuring 1–8 μm in length (Figs. 2, 3).

Spread granulocytes are motile and portray active cyclosis. Occasionally, granulocytes, which appear to have fused with one another, have been observed in preparations of living cells (Fig. 5). The resulting giant, multinucleate cells contain the same cytoplasmic inclusions as those occurring in individual granulocytes. Cyclosis also occurs in these multinucleate cells.

Fibrocytes. Cells of this type are generally spindle-shaped in the contracted state and subsequent to adherence, spread slowly across the substrate with the nucleus usually located along one side (Fig. 6).

These fibrocytes contain motile vermiform bodies (possibly mitochondria), each measuring up to 2 μm in length and which are more or less evenly distributed throughout the cytoplasm (Fig. 7). Small refractile bodies (< 1 μm) generally occur adjacent to the nucleus. In this type of cell the demarcation between the endoplasm and ectoplasm is not as well defined as in granulocytes.

Beyond the periphery of the spread fibrocyte protrude a variable number of filopodial projections, which also can be traced back into the endoplasm (Fig. 6).

Following spreading, small sections of cytoplasm are occasionally detached from the substrate and contract or undulate, thus resulting in the slow movement of the cell across the slide. Cyclosis occurs in spread fibrocytes.

Hyalinocytes. The hyalinocyte spreads slowly outward over the substrate. These cells contain some vermiform bodies (possibly mitochondria), vacuoles, few refractile bodies situated near the large, compact, eccentrically located nucleus, and few or no filopodial projections. Slow cyclosis occurs. The hyalinocytes appear to be the least motile of the three cell types.

Uptake of vital dyes

Granulocytes. The cytoplasmic inclusions designated herein as granules take up neutral red from a concentration of 0.02% within 15 seconds; however, the refractile inclusions do not take up the dye. Neutral red in the concentration employed does not appear to be deleterious to these cells. When exposed to

Janus Green B at a concentration of 0.002%, nearly all of the cytoplasmic granules take up the dye rapidly. Furthermore, after 15 minutes, some granules appear violet or purple, thus indicating possible reduction of this dye to diethyl safranin. The refractile cytoplasmic inclusions also do not take up this dye. When exposed to a mixture of neutral red and Janus Green B at a final concentration of 0.001% for each stain, the cytoplasmic granules appear red. It is noted that there are only a few cytoplasmic inclusions, which are interpreted to be mitochondria, that take on the bluish color imparted by Janus Green B.

Fibrocytes and hyalinocytes. Except in the case of a few isolated cytoplasmic inclusions, both of these types of cells do not take up appreciable amounts of either neutral red or Janus Green B, singly or combined, at the concentrations employed.

TABLE IV

Mean dimensions of spread, fixed, and stained leucocytes of Mercenaria mercenaria *(n = 100 for each cell type from each group). All measurements are given in µm.*

	Mean cell length	Mean cell width	Mean nuclear length	Mean nuclear width
		Great Bay		
Granulocytes	x̄ = 35.41	25.71	6.63	4.76
	sd = 7.66	6.38	0.94	0.96
Fibrocytes	x̄ = 27.42	20.54	6.41	4.41
	sd = 7.13	5.78	1.54	0.98
Hyalinocytes	x̄ = 26.18	21.36	7.87	6.00
	sd = 6.84	5.19	1.51	1.05
		Buzzard's Bay		
Granulocytes	x̄ = 32.87	25.96	6.24	4.57
	sd = 7.36	6.75	1.19	0.78
Fibrocytes	x̄ = 27.14	20.41	5.82	4.09
	sd = 7.22	5.24	1.32	0.95
Hyalinocytes	x̄ = 25.54	21.30	7.60	5.79
	sd = 6.28	5.20	1.47	1.04

Staining characteristics

Examination of glutaraldehyde-fixed and Giesma-stained, spread leucocytes verified the presence of the three cell types as based on their nuclear and cytoplasmic morphology and on the ratios of nuclear to total cell areas (Tables IV and V). These distinguishable cell types correspond to the three recognizable in preparations of living cells. The dimensions and differential analyses of the three cell types are presented in Tables III and IV, respectively. The ratios of nuclear to total cell areas are listed in Table V.

Granulocytes. The endoplasm of spread, stained granulocytes contains vacuoles, an eccentrically situated, compact nucelus, and pale blue or pink cytoplasmic granules in a network of intensely stained small (< 1 µm) blue inclusions (Fig. 9).

The pale blue ectoplasm is very finely granular and includes a variable number of spike-like projections, many of which appear to originate in the endoplasm (Fig. 9). Occasionally, granulocytes with multiple nuclei are observed (Fig. 10).

Fibrocytes. The cytoplasm of fibrocytes contains an interlacing, loose array of fibrous material that is most conspicuous in the ectoplasm. Small blue inclusions are situated at the junction of these fibers. The endoplasm is slightly vacuolar and contains the compact, eccentrically situated nucleus. From the periphery of the fan-shaped ectoplasm spread a variable number of spike-like projections, which in some instances originate in the endoplasm (Fig. 11).

Hyalinocytes. The vacuolar, pale-blue cytoplasm is spread subcircularly around the eccentrically located, large oval nucleus. The endoplasm stains more darkly than the ectoplasm and a diffuse gradient occurs between the two zones. Few or no projections occur along the periphery (Fig. 12).

TABLE V

Ratios of mean nuclear area to mean total cell area of fixed and stained spread leucocytes from two groups of Mercenaria mercenaria *(n = 100 cells of each type from each group); z tests, degrees of freedom (df) = 99, $P < 0.01$; Great Bay: $A = B$, $z = 5.94$; $A = C$, $z = 12.19$; $B = C$, $z = 7.63$. Buzzard's Bay: $A = B$, $z = 3.63$; $A = C$, $z = 12.65$; $B = C$, $z = 7.86$. z test for same type of cells from the two groups: $df = 99$, $P > 0.05$. Granulocytes, $z = 0.60$; fibrocytes, $z = 1.44$; hyalinocytes, $z = 0.57$.*

	Great Bay	Buzzard's Bay
A. Granulocytes	$\bar{x}$ = 0.0387	0.0373
	sd = 0.0169	0.0163
B. Fibrocytes	$\bar{x}$ = 0.0567	0.0507
	sd = 0.0253	0.0331
C. Hyalinocytes	$\bar{x}$ = 0.0944	0.0911
	sd = 0.0426	0.0393

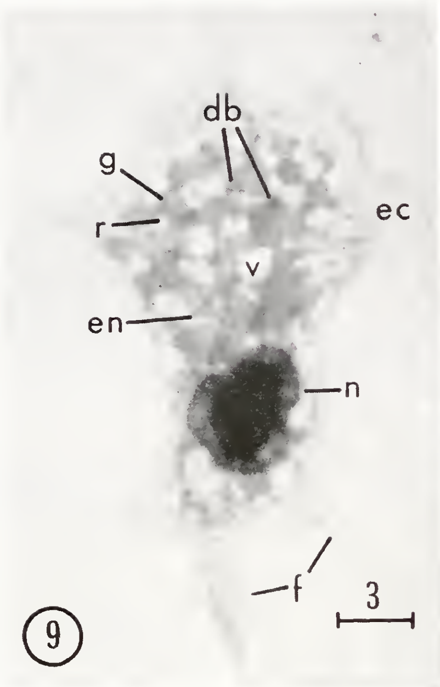
DISCUSSION

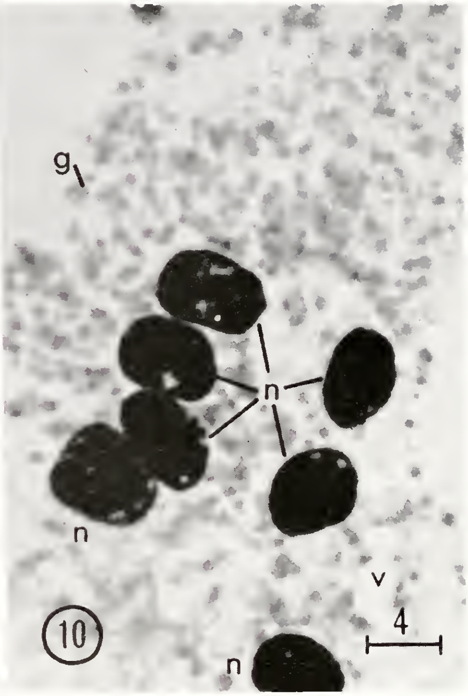
Although several earlier studies on the leucocytes of *M. mercenaria* are available (Zacks, 1955; Zacks and Welsh, 1953; Janoff and Hawrylko, 1964), no attempt has been made thus far to classify or distinguish between the morophological types of leucocytes found in the hemolymph of this clam or to determine whether or not hemolymph cells from *M. mercenaria* from different geographic locations are similar. Instead, the earlier investigators have been concerned with the most conspicuous type of cell, the granulocyte, and have largely ignored the other types of cells present in the hemolymph.

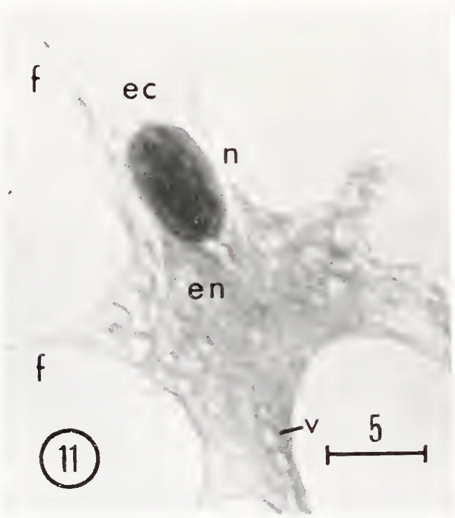
The results reported herein indicate the occurrence of three morphological types of leucocytes. In addition, it is now known that these leucocytes are similar, both qualitatively and quantitatively, in clams taken from two geographic locations (Tables IV and V).

Because the clams used in this study were all very similar in size, we feel that it is valid to compare hemolymph cells from both groups of clams.

The high variability in total cell counts undoubtedly reflects differences in the total numbers of circulating leucocytes in individual clams. Previous studies sup-







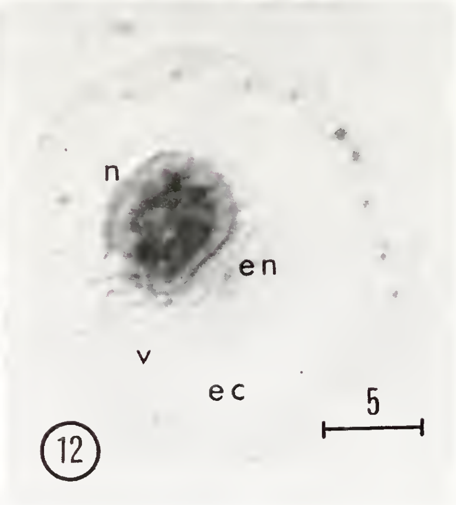


FIGURE 9. Figures 9–12 are fixed and stained leucocytes of *Mercenaria mercenaria.* All cells were fixed with glutaraldehyde except that shown in Figure 10 which was fixed with methanol. Scale-bar units are in μm; Figure 9, granulocyte with filopodia-like projections (f) showing ectoplasm (ec), endoplasm (en) containing refractile bodies (r), vacuoles (v), nucleus (n), and granules (g) with blue inclusions (db) between them (Giemsa stain).

FIGURE 10. Multinucleate granulocyte with seven visible nuclei (n), cytoplasmic granules (g), and vacuoles (v) (Giemsa stain).

port this hypothesis. Specifically, Feng (1965a) has observed that in another estuarine pelecypod, *Crassostrea virginica,* the number of circulating leucocytes is dependent upon the amount of turbulence produced by cardiac action. In addition, he has proposed that numbers of leucocytes may also vary with the feeding and excretion cycles since leucocytes participate in both of these activities. It is thus of interest to note that Feng (1966b) has determined that there is a diurnal cycle of feeding activity in *M. mercenaria* and that while this cycle can be disrupted by changes in intensity of light, temperature, and salinity, or by the presence of organic materials, individual clams may remain inactive, *i.e.*, not pumping, for several days. Thus, variability of circulating leucocytes in the clams may be tentatively attributed to differences in the physiological state of the individuals.

Coefficients of correlation computed between the dimensions of each individual clam and the differential counts, total cell counts, and packed cell volumes are all very small. In addition, coefficients of correlation comparing number of granulocytes with packed cell volumes and total cell counts are also very small. It appears, therefore, that these hemotologic characteristics of leucocytes are independent of the size of the individual clam and are also independent of one another.

As noted, the ratios of nuclear to total cell areas (Table V) are approximate but are included to provide a relative comparison of cell spreading and nuclear size among the three cell types in both groups of clams. With this consideration in mind, it is noted that the means of these ratios for the three cell types within each sample are significantly different using the z test as presented by Tate (1965) ($P < 0.01$), while the means of ratios for cells of the same type are not significantly different using the same test ($P > 0.05$) (Table V). From these observations it is possible to draw certain conclusions. First, the same cell types from clams collected from different areas appear to have the same nuclear size and spreading characteristics, and therefore, we assume that the same leucocytes occur in specimens of *M. mercenaria* from all different geographical populations. In addition, the extreme separation of the means of the ratios obtained from the different cell types suggests that these three cell types are distinct (Table V). However, until further experiments designed to answer questions relative to the origin and ontogeny of molluscan leucocytes are conducted, no definitive statement can be made pertaining to the interrelationships between the granulocytes, hyalinocytes, and fibrocytes of *M. mercenaria.*

Regarding the vital staining of inclusions in leucocytes, Zacks and Welsh (1953) and Zacks (1955) have stated that those inclusions in the granulocytes of *M. mercenaria* that Zacks (1955) has designated as "specific granules," are atypical mitochondria. Zacks (1955) based this assumption on three tests: (1) the uptake of Janus Green B, a traditional mitochondrial vital stain; (2) a positive reaction for phospholipids; and (3) the presence of dehydrogenase activity as determined by the Nitro Blue Tetrazolium test. Zacks also has stated that those vacuoles in

FIGURE 11. Fibrocyte with filopodia-like projections from the ectoplasm (ec), and containing nucleus (n), and vacuoles (v) in the endoplasm (en) (Nomarski interference; Giemsa stain).

FIGURE 12. Hyalinocyte showing ectoplasm (ec) with no filopodia-like projections; and endoplasm (en), with nucleus (n), and a few vacuoles (v) (Nomarski interference; Giemsa stain).

leucocytes that take up neutral red are not the same as the granules that take up Janus Green B. His opinion was based on differences in the appearance of these organelles. Zacks has also reported that the neutral red vacuoles are not preformed in the granulocyte, but form as the dye accumulates in the cells.

To the contrary, on the basis of our observations on the uptake of these two vital dyes by the granulocytes of *M. mercenaria*, we find that the inclusions referred to earlier in this paper as granules, which we believe to be identical to those designated by Zacks (1955) as "specific granules," take up both Janus Green B and neutral red. We have not observed a separate, large population of granules selectively stained with Janus Green B when a mixture of the two stains was used. Thus, we are presently of the opinion that there is only one type of cytoplasmic granule in *M. mercenaria* and it is stained with both Janus Green B and neutral red.

Relative to the selective settling of molluscan leucocytes, Prowse and Tait (1969) have reported that only certain leucocytes of *Helix aspersa* adhere to glass. However, we have found as a result of differential counting of *M. mercenaria* leucocytes in fresh and fixed hemolymph preparations on slides where the cells had been permitted to adhere prior to fixation and staining, that the results are approximately the same (Table III). It thus must be concluded that cells of all three types are capable of adhering to glass.

Leucocytes not only adhere to glass but also to one another. Although there appears to be no true coagulation in the hemolymph of *M. mercenaria*, agglutination of the leucocytes occurs *in vitro* and is more pronounced if the leucocytes are mixed with seawater or with homologous serum. Agglutination observable to the naked eye commonly occurs in these cases. Microscopical observations have shown that leucocytes that make contact *in vitro* will generally adhere to one another.

Also associated with the phenomenon of cell agglutination is the occasional fusion of granulocytes to form multinucleate cells. That true fusion does occur is supported by the observation that during cyclosis the granules and nuclei from all of the participating cells move in the large cytoplasmic mass. The occurrence of multinucleate cells has been reported in *Crassostrea gigas* by Sparks and Pauley (1964) and in *Helisoma duryi normale* by Cheng and Galloway (1970). In both of these instances however, the molluscs had been subjected to injury.

Relative to the adherence and spreading of leucocytes, it is possible that the spike-like processes formed with fans of ectoplasm stretched between them may serve a protective function. These processes generally occur on granulocytes and only rarely on hyalinocytes. Earlier, Bang (1961) had reported that the filopodial processes of leuocytes of oysters are responsible for the capture of microorganisms. Such microorganisms, according to Bang, also appear to be trapped by sticking in the nets of ectoplasm. A similar function may occur in the case of clam leucocytes.

With the establishment of three distinct types of cells in the hemolymph of *M. mercenaria* and having quantified some of the hematologic parameters, it is now possible to attempt to answer some important basic questions relative to the role of these cells in internal defense against foreign materials.

We would like to thank Frank W. Koko, Jr., co-author of the LEAPS program, for his valuable assistance with the statistical analyses.

SUMMARY

The hematological parameters of *Mercenaria mercenaria* of similar size from two geographical areas as well as the morphology and behavior of their leucocytes were studied. On the basis of qualitative and quantitative characteristics, three types of leucocytes, designated as granulocytes, fibrocytes, and hyalinocytes, can be distinguished in both living and stained preparations.

A correlation matrix computed between all parameters considered has revealed an insignificant correlation between the dimensions of whole animal and the differential count, packed cell volume, and total cell count as well as an insignificant correlation between the hematological parameters themselves. The only exception is that there is a positive correlation between the packed cell volume and the total cell count.

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PITUITARY CONTROL OF ADAPTATION TO FRESH WATER IN THE TELEOST GENUS *FUNDULUS*

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Despite intensive investigation in recent years of the phenomenon of hypophysial control of teleostean osmoregulation in fresh water by prolactin (see reviews by Ball, 1969a, 1969b; Ensor and Ball, 1972; Lam, 1972), several aspects of this problem have remained little explored. Burden (1956) reported that hypophysectomized *Fundulus heteroclitus* cannot survive indefinitely in half strength Long Island Sound water (salinity 13‰), a medium somewhat hyperosomotic to the blood, although they can in full strength Sound water. This is difficult to reconcile with the widely-held assumption that failure of hypophysectomized fish is due solely to an inability to maintain normal internal electrolyte levels in the face of an electrolyte-poor environment. The effect of temperature on the failure of hypophysectomized fish in fresh water has been little studied, although it is well known that temperature has a profound effect on osmoregulation in fishes (Kinne, 1967). Broad comparisons of survival after hypophysectomy have been made between species (Schreibman and Kallman, 1966) despite the fact that experimental temperatures differed between many of the studies cited. Pickford, Pang, Stanley and Fleming (1966) found that high environmental calcium levels permit *Fundulus zebrinus* (= *F. kansae*, *cf.* Drewry, 1967) to survive in fresh water after hypophysectomy but had no observable effect on the failure of *F. heteroclitus*. Ball (1969b) noted that another species of this genus, *F. diaphanus*, could survive after hypophysectomy for long periods in low calcium fresh water.

Investigations on the control of osmoregulation by the pituitary in teleosts have led to speculation on the implications of this phenomenon in terms of vertebrate evolution (Bern, 1967) and more specifically in terms of teleost evolution (Schreibman and Kallman, 1966, 1969). The latter authors, studying poeciliid fishes and some of their relatives, but making broader generalizations from data published on other species, have suggested that hypophysial control of freshwater tolerance may be of limited taxonomic distribution among teleosts and that normal environmental salinity has little influence on pituitary-dependent survival in fresh water. These conclusions must be regarded with some caution, however, as it appears that survival may be a misleading index of the ionoregulatory economy of fish (Ball, 1969a), and studies on the hypophysial control of osmoregulation in teleosts are limited to far too few species for confident phylogenetic or ecological generalizations.

In light of questions surrounding some physiological aspects of hypophysial control of adaptation to dilute media and uncertainties regarding the respective contributions of environmental and phylogenetic factors to dependence on the pituitary for survival in fresh water, we have chosen to investigate the role of the pituitary in survival of killifishes of the genus *Fundulus* in fresh water. The genus *Fundulus,* containing some 26 mainland North American species, is ideal for such studies. It includes 9 brackish-water species with varying affinities for fresh water, 8 freshwater species with coastal distributions which may, on occasion, enter brackish water, 8 species geographically isolated in fresh water, and one inland species commonly found in saline water. In addition, the brackish water mummichog, *F. heteroclitus,* is perhaps the most thoroughly known teleost in terms of hypophysial control of adaptation to fresh water.

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We have conducted a survey of 23 species and one subspecies of *Fundulus* and one species of the related Middle American genus *Profundulus* in regard to the ability to survive in fresh water after hypophysectomy. It is hoped that such an approach on a taxonomically unified but environmentally-diverse group of fishes will provide insight into the interplay between phylogenetic and ecological factors involved in the determination of the importance of the pituitary in adaptation to fresh water. In addition, we have attempted to clarify the influence which experimentally-modifiable environmental parameters (salinity, calcium, and temperature) have on failure. Such data are necessary to a rational comparison of the failure times of species whose natural environments may differ in regard to temperature range and other factors. Furthermore, we have undertaken to compare the response of blood electrolytes to hypophysectomy in different species and under different laboratory conditions in an attempt to elucidate the physiological basis for failure, and to ascertain whether it is homologous between species and within a species under different environmental conditions.

Materials and Methods

The nomenclature used in the present study generally follows that of Miller (1955). The nominal *F. kansae* and *F. similis* are regarded as subspecies of *F. zebrinus* and *F. majalis* respectively following Drewry (1967) and Relyea (1967). *F. swampinus* is recognized as a valid species following Rivas (1966, as *F. lineolatus*) and Griffith (1972). The unnamed species from Tennessee is that species referred to as the "barrens topminnow" by Miller (1972), and formerly relegated to *F. albolineatus* (Miller, 1955; Brown, 1957).

A total of 14 freshwater species of *Fundulus*, 8 species and one subspecies of brackish-water *Fundulus*, and one inland species of the genus from saline water were collected between 1968 and 1970 from their normal habitats in the eastern, southern, mid-western and Pacific coastal United States. Briefly, the brackish-water species include *F. confluentus*, *F. jenkinsi* and *F. pulvereus* from low salinity brackish environments, *F. heteroclitus* and *F. grandis* found in widely variable salinities, and *F. luciae*, *F. majalis* and *F. parvipinnis* typical of high salinities. *F. zebrinus* is an inland species found in both fresh and saline waters. Among the freshwater species, *F. cingulatus*, *F. notti* and *F. swampinus* are normally found in dilute swamp water, *F. rathbuni*, *F. stellifer* and *F. waccamensis* in fresh waters of moderate ion content, *F. catenatus*, *F. sciadicus*, *F. seminolis* and the undescribed species in fresh waters with high concentrations of dissolved minerals, and *F. chrysotus*, *F. diaphanus*, *F. notatus*, and *F. olivaceus* are widespread species which inhabit fresh waters of varied salt content. Although *F. chrysotus*, *F. cingulatus*, *F. diaphanus*, *F. notatus*, *F. notti*, *F. olivaceus*, *F. semi-*

nolis, and *F. swampinus* are all distributed in coastal regions, only *F. diaphanus* and *F. chrysotus* have been reliably reported to enter dilute brackish water environments. Further details on environmental salinities of all species are given elsewhere (Griffith, 1974).

The collection sites are generally identical to those tabulated by Griffith (1974) with the following exceptions: the *F. catenatus* in the present study included specimens from both Osage (group A) and Wright (group B) Counties, Missouri; the *F. olivaceus* were from Holmes Co., Florida (group A) and Cape Girard Co., Missouri (group B); the *F. rathbuni* were from Orange (group A) and Mecklenberg (group B) Counties, North Carolina; and the *F. swampinus* were from Columbus Co., North Carolina (group A) and Leon Co., Florida (groups B and C). Specimens of *Profundulus punctatus* were tank-raised individuals provided by Dr. Neal R. Foster (Philadelphia Academy of Natural Sciences).

For most species, young adult specimens were studied, but in the cases of *F. heteroclitus* (group B), *F. jenkinsi, F. catenatus, F. olivaceus* (group B), *F. stellifer* and *F. waccamensis* (groups B and C) juveniles were used. The groups investigated generally included both sexes although only males were studied in *F. heteroclitus* (groups A and B), *F. majalis* (group D), *F. diaphanus* (group A), *F. swampinus* (group B) and *F. seminolis,* whereas only females were tested for *F. heteroclitus* (group D) and the undescribed species.

Upon arrival in New Haven, Connecticut, freshwater species were initially adapted to running, dechlorinated New Haven tap water (salinity less than 0.1‰, calcium less than 8 ppm) and brackish or saline species were adapted to recirculated sea water (Long Island Sound water, salinity 29‰). The fish were fed a diet of modified Aronson's mixture (Pickford, 1953) liberally supplemented with frozen brine shrimp and were maintained on an 8 hour day. After laboratory acclimation of at least one month, fish were hypophysectomized by the opercular method of Pickford (1953), modified for smaller fish by cutting along the base of the operculum. Hypophysectomy of freshwater species was performed in dilute sea water (salinity 6‰), found empirically to be the medium at which recovery was optimal. Brackish-water or saline species were hypophysectomized in either sea water or dilute sea water (6, 10 or 12‰) and were permitted to recover in this medium for a period varying from a week to several months; the duration being brief in the case of dilute sea water which some brackish-water species cannot tolerate indefinitely after hypophysectomy (Burden, 1956). Post-operative mortaility prior to testing was negligible for most species.

After 8–14 days of recovery in the operation medium, freshwater species were tested by abrupt transfer to tap water. Unoperated controls were maintained continually in tap water. Hypophysectomized and unoperated brackish-water species were tested both by abrupt transfer from the operation medium to fresh water and by gradual dilution of the salinity of the home tank by allowing tap water to slowly drip in. For the latter tests, salinity declines of 1.0 to 1.8‰ per day were achieved. The symptoms of failure for both freshwater and brackish-water species were as described by Burden (1956). Loss of balance was regarded as the definitive criterion of failure. Transfer back to sea water or dilute sea water was attempted for many species after failure had occurred, and was generally successful for brackish-water species but only occasionally for freshwater species.

Completeness of hypophysectomy was judged on the bases of initial notes on the operation, failure of the fish to increase in length, abnormal fin regeneration, pallor, gonadal regression and post mortem dissection. While it is recognized that there are reports of small clusters of prolactin-secreting cells left after imperfect hypophysectomies proliferating and permitting survival in fresh water (Pickford, Robertson, and Sawyer, 1965; Ball, 1965a, 1965b; Schreibman and Kallman, 1969), and that regeneration of that portion of the pituitary involved in osmoregulation need not be accompanied by regeneration of other portions, it is most unlikely that this could be the case in the vast majority of specimens in the present study in which the pituitary was observed to be removed as an intact organ. At least one of the collateral parameters on which completeness of hypophysectomy was judged (pallor) is primarily a function of the same hormone responsible for freshwater survival in *F. heteroclitus*. In this species melanogenesis is under prolactin control while only the proliferation of new melanophores is affected by intermedin (Pickford and Kosto, 1957). Specimens for which there was any doubt regarding the completeness of hypophysectomy have not been considered in our study.

In addition to studies of survival in fresh water for all species of *Fundulus*, we have assessed the roles of environmental calcium and salinity on the failure time of *F. heteroclitus*. For the former experiment, water with a calcium concentration of 370 ppm was obtained by adding $CaCl_2$ to tap water. For the latter studies, sea water was diluted with tap water to obtain salinities of 1, 3, and 5‰ in one experiment and 1, 6, and 12‰ in a second. The effect of temperature (20°, 15°, 8°, and 1° C) was tested on the freshwater failure of *F. heteroclitus*. In this study the specimens were hypophysectomized in sea water at 15° C and were then acclimated to the test temperature for at least one month prior to testing. While most other species were tested only at 15° C, several (*F. luciae*, *F. chrysotus*, *F. olivaceus*, *F. swampinus* and *F. waccamensis*) were tested at both 15° and 20° C, and two (*F. notti* and *Profundulus punctatus*) were tested at only 20° C. 15° C was chosen as the principal experimental temperature to minimize problems of sexual activity in control fish and infection in the specimens, and also to facilitate comparison with other studies on *Fundulus*, most of which have been conducted at 15° C. All populations of *Fundulus* tested, with the possible exceptions of *F. parvipinnis* from Southern California and *F. confluentus* from peninsular Florida, were collected in geographical areas in which environmental water temperatures well below 15° C are normal during the winter.

Blood electrolyte levels were measured in two brackish-water species (*F. majalis* and *F. heteroclitus*) and in three freshwater species (*F. diaphanus*, *F. swampinus* and *F. rathbuni*). Blood was collected from intact and hypophysectomized *F. heteroclitus* in sea water, hypophysectomized fish failing in fresh water, dilute sea water on high calcium fresh water, and mock-operated controls sacrificed after 12 days in fresh water or high calcium fresh water. Intact and hypophysectomized *F. majalis* were studied in sea water and failing in fresh water. Blood electrolyte studies on the three freshwater species were conducted on fish hypophysectomized in dilute sea water (6‰), transferred to fresh water 8–14 days after the operation, and sacrificed after a period of time in this medium (29 days for *F. rathbuni*, 31 days for *F. swampinus*, and at 8 and 48 days for *F. diaphanus*). Blood from specimens failing in fresh water was also collected for *F. rathbuni* and *F. diaphanus*, and blood of intact and hypophysectomized *F. diaphanus* was collected after one week in sea water following a very gradual increase in salinity. For the blood studies, free-flowing blood was collected in non-heparinized microhematocrit tubes from the severed caudal penuncle (or by heart puncture in the case of *F. swampinus*), permitted to clot, centrifuged, and 5 μl aliquots of serum were used for the determination of chloride (Amino-Cotlove chloride titrator) and sodium and potassium (Instrumentation Laboratory flame photometer) following procedures described in Pickford, Grant and Umminger (1969).

Table I

Effect of temperature, salinity and calcium on the survival of hypophysectomized F. heteroclitus

Group	Pre-transfer medium		Experimental medium		N	Hours until failure	
	Salinity	Days adapted	Salinity	Temperature		Mean ± S.E.	Range
A1	29‰	105	FW	15°	3	129 ± 18	94–152
B1	29‰	48	FW	20°	7	79 ± 22	10–117
B2	29‰	48	FW	15°	7	86 ± 21	19–166
B3	29‰	48	FW	8°	9	232 ± 35*	126–476
B4	29‰	48	FW	1°	9	251 ± 45*	117–476
B5	29‰	30	1‰	20°	7	137 ± 16	68–187
B6	29‰	30	3‰	20°	6	359 ± 9†	334–388
B7	29‰	30	5‰	20°	8	246±46†	101–428
C3	29‰	30	FW	20°	7	146 ± 21	96–264
C5	29‰	30	1‰	20°	6	451 ± 73††	194–622
C6	29‰	30	6‰	20°	5	504 ± 164††	264–1152
C7	29‰	30	12‰	20°	6	787 ± 209††	271–1326
C4	29‰	30	FW + Ca^{++}	20°	6	166 ± 17	125–228

* Significantly different from both 15° and 20°, $P < 0.01$.
† Significantly different from appropriate freshwater controls (group B1), $P < 0.01$.
†† Significantly different from freshwater controls, $P < 0.01$.

The statistics presented in this paper are means ± standard errors calculated for small sample numbers and statistical significance, when appropriate, was determined using Student's *t* test. For several species, difficulties in collection or transportation prevented the study of adequate numbers of specimens for proper statistical analysis. Such data have been included for the sake of completeness, although it is recognized that small numbers may preclude very strong interpretations.

Results

Effect of temperature on survival in fresh water after hypophysectomy

Data is presented in Table I demonstrating the effect which adaptation temperature has on failure time in hypophysectomized *Fundulus heteroclitus* in fresh water. In this species low temperatures retard failure. Survival time at high temperatures (groups B1 at 20° and B2 at 15° C) is roughly one-third that at low temperatures (groups B3 at 8° and B4 at 1° C). There is little difference in any of the experiments between failure time at 15° and 20° C in *F. heteroclitus*, and there also appears to be little difference between 8° and 1° C; a discontinuity between long and short survival seems to occur between 15° and 8° C in this species. We might note that while we found no significant difference in time until failure between 15° and 20° C, a major difference was found in successful recoveries of failing fish returned to sea water. At 20° C only 29% of the failing fish could be saved. At 15°, 8°, and 1° C the respective survivals were 71%, 89%, and 77%. It would appear that the progression of osmoregulatory failure after the first symptoms appear is more rapid at the higher temperature.

TABLE II

Comparison of survival of brackish-water species of Fundulus *in fresh water after hypophysectomy*

Species	Group	Pre-transfer medium		Temperature	Number	Hours until failure	
		Salinity	Days adapted			Mean ± S.E.	Range
F. confluentus	A1	29‰	12	15°	1	57	
	A2	10‰	10	15°	1	106	
F. grandis	A1	29‰	17	15°	3	137 ± 39	94–215
F. jenkinsi	A1	10‰	10	15°	4	200 ± 20	150–240
	A2	29‰	9	15°	4	197 ± 45	148–330
F. luciae	A1*	6‰	14	20°	7	23 ± 0	23
	A2*	6‰	16	15°	5	35 ± 5	25–47
	A3*	12‰	5	15°	2	43	43
F. majalis majalis	B1*	29‰	64	15°	8	18 ± 7	9–70
	C1*	12‰	1	15°	5	24 ± 4	21–30
	D1*	6‰	7	15°	3	57 ± 21	25–97
F. majalis similis	A1	10‰	13	15°	2	111	94–127
	A2	29‰	10	15°	1	45	
F. pulvereus	A1	10‰	10	15°	3	139 ± 20	92–153
	A2	29‰	9	15°	2	126	103–148
F. zebrinus	A1	FW + Ca^{++}	30	15°	2	372	144–600

* Some failure observed in intact controls in fresh water. See text for details.

A number of species in addition to *F. heteroclitus* were tested at both 15° and 20° C. In the brackish-water species, *F. luciae*, high temperature accelerates failure in both intact and hypophysectomized fish. Under conditions of rapid transfer from dilute saline (6‰) to fresh water, the intacts fail within 5–8 days at 20° and survive for over a month at 15° C, while hypophysectomized fish fail within one day at 20° and between one and two days at the lower temperature (Table II). Among the freshwater species there appears to be some acceleration of failure at 20° in *F. olivaceus, F. swampinus* and *F. waccamensis,* while in *F. chrysotus* there is, if anything, longer survival at the higher temperature (Fig. 1). There is no apparent correlation of the effect of temperature on freshwater failure with geographic latitude or with environmental salinity. The opposing data on the freshwater species *F. chrysotus* and *F. swampinus* were from fish collected within a few miles of each other and the same is true of the data on the brackish-water *F. heteroclitus* and *F. luciae* tested at 15° and 20° C.

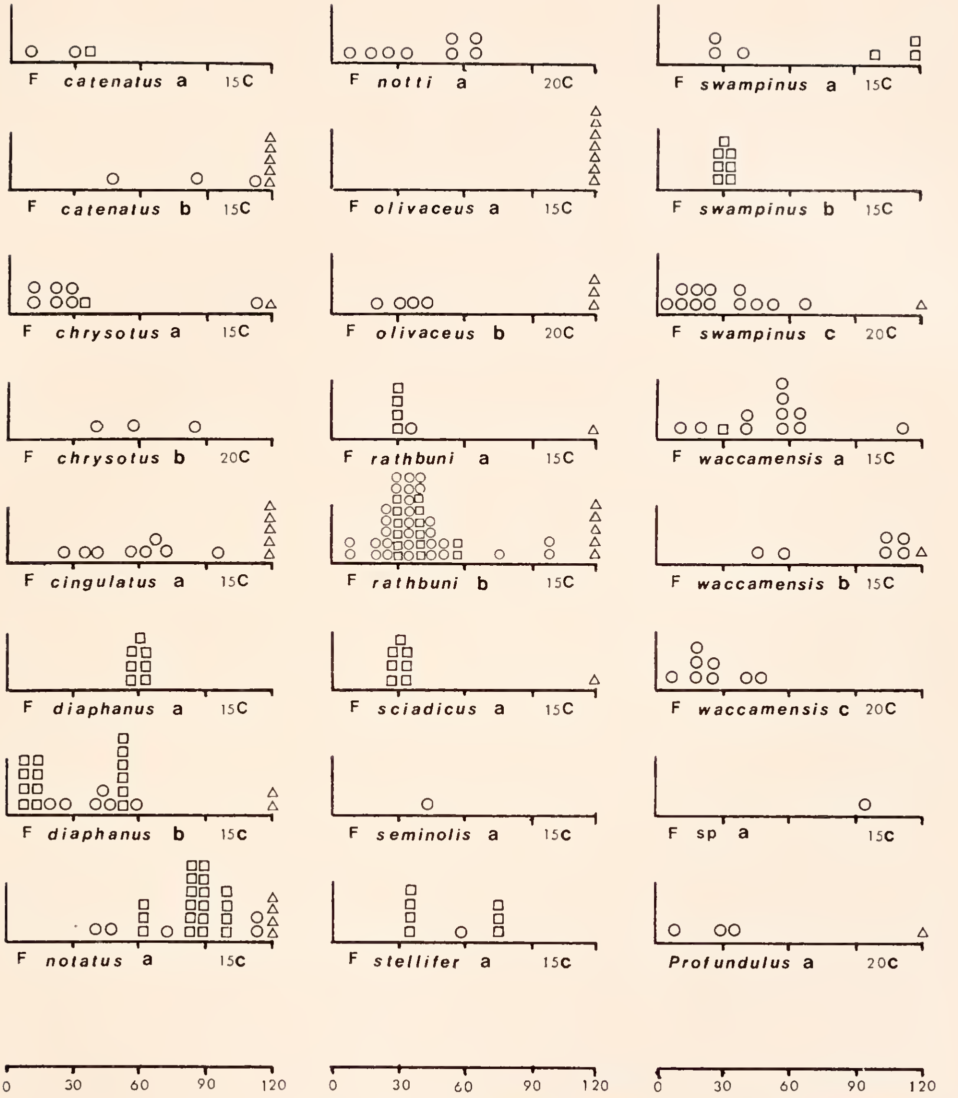

FIGURE 1. Survival of hypophysectomized freshwater species of *Fundulus* and *Profundulus* in fresh water. The number of days since transfer to fresh water (horizontal axis) is given at the bottom. Failing fish are depicted as circles, fish removed from the experiment as squares, and fish surviving over 120 days as triangles. Group designation and experimental temperature are shown for each species.

A consideration of published reports on the failure of *F. heteroclitus* indicates that studies conducted at 15° (Burden, 1956; Pickford, Robertson and Sawyer, 1965) give results in agreement with ours, while Potts and Evans (1966) reported failure times of less than one day at 20° C, in contrast to our data which indicate failure time at this temperature of 3–6 days. Potts and Evans adapted their fish to 40% sea water, a medium in which hypophysectomized *F. heteroclitus* cannot survive indefinitely (Burden, 1956), and it is possible that the fish studied by these authors were already near failure at the time they were transferred to fresh water. Our demonstration that low temperature generally prolongs survival after hypophysectomy of species of *Fundulus* in fresh water is in accord with the results of Chidambaram, Meyer and Hasler (1972) on *Ictalurus melas.*

Effects of environmental salinity and calcium on survival of F. heteroclitus

Table I presents data on the influence of salinity (compare groups B1, B5, B6, and B7 for experiment one and C3–C7 for the second) and calcium (groups C3 and C4) on the survival of hypophysectomized *F. heteroclitus* at 20° C. In both salinity experiments the addition of sea water to the tank significantly prolonged survival over that of fish in regular tap water. In the second experiment a progressive increase in survival time was found with increasing salinity although this was not clear in the first experiment. Failure was even observed at 12‰, a salinity at which the calculated osmolality (366 mOsm/kg) is essentially identical to serum osmolality of intact *F. heteroclitus* in sea water (Pickford *et al.*, 1969). This observation confirms the studies of Burden (1956) who reported that hypophysectomized *F. heteroclitus* fail at 13‰, and of Pickford *et al.* (1965) who found that the failure of hypophysectomized *F. heteroclitus* subjected to gradual decreases in salinity was indicative of an initiation of the process at salinities near those observed by Burden (1956) to lead to eventual failure. We should note here that the somewhat shorter survival observed for the groups in the first experiment may be attributable to size differences. In the first investigation very small juveniles (1–2g) were used, while for other experiments on *F. heteroclitus* young adults (generally over 4g) were studied.

We failed to find any prolongation of survival of hypophysectomized *F. heteroclitus* when high levels of calcium (370 ppm Ca^{++} as $CaCl_2$) were added to tap water. The fact that in the same experiment survival was significantly prolonged at 1‰, a salinity at which chloride levels are lower and osmolality only slightly higher than the calcium-rich tap water, suggests that the beneficial effect of dilute sea water on survival of hypophysectomized *F. heteroclitus* is likely due to sodium concentration. Our negative findings on the effect of calcium on *F. heteroclitus* confirm the data of Pickford, Pang, Stanley and Fleming 1966 on this species, and contrast with the positive results of the same authors on *F. zebrinus.*

Comparison of survival of hypophysectomized Fundulus *species and* Profundulus *in fresh water*

Brackish-water species of *Fundulus* tested by rapid transfer from sea or dilute sea water to fresh water demonstrate mean times until failure ranging from about one day in *F. majalis* and *F. luciae* to eight days in *F. jenkinsi* (Table II). Data on survival of intact control specimens has been presented elsewhere (Griffith, 1974) and, because failure of such specimens was the exception, are not repeated in the tables. When failure did occur it is noted in the text. Specimens of *F. zebrinus,* an inland species frequently found in saline water, fail after about two weeks when transferred from high calcium fresh water to normal tap water. The remaining brackish-water species show mean survival of 2–5 days at 15° C. There appears to be no consistent effect of the pre-adaptation medium on failure in the species tested. In *F. majalis* a period in dilute sea water has an ameliorative effect, but this appears not to be the case with some of the other species. Failure of intact *F. luciae* was observed at 20° but not at 15°. *F. majalis* intacts failed at the same time as hypophysectomized fish when subjected to abrupt transfer from sea to fresh water but survived well when permitted to adapt to dilute sea water (6‰) prior to testing. With the exception of *F. parvipinnis*, intacts of which fail within a day of transfer to fresh water from sea water but hypophysectomized specimens were not tested by this procedure, failure was not observed in intact controls of the other species.

Table III

Salinity at failure for hypophysectomized brackish-water species of Fundulus *subjected to a gradual decrease in salinity at 15° C. Values in brackets exclude a single abnormally high fish.*

Species	Group	Number	Rate of decrease in ‰/day	Salinity at failure (‰)	
				Mean ± S.E.	Range
F. zebrinus	A	6	1.1	0.95 ± 0.65 [0.30 ± 0.09]	0.1–4.2 [0.1–0.5]
F. jenkinsi	A	3	1.4	3.20 ± 0.35 [0.35]	0.3–8.9 [0.3–0.4]
F. grandis	A	8	1.1	1.33 ± 0.05†	1.1–1.5
F. confluentus	A	5	1.2	1.54 ± 0.14	1.3–1.9
F. heteroclitus	A	5	1.0	1.64 ± 0.07	1.2–2.0
F. luciae	A	8	1.3	3.44 ± 1.22* [1.36 ± 0.33]	0.4–18.0 [0.4–3.3]
F. majalis	A	3	1.3	3.10 ± 0.35*	2.6–3.8
F. m. similis	A	3	1.2	3.22 ± 1.23	0.9–5.0
F. pulvereus	A	3	1.4	3.40 ± 0.10	3.2–3.5
F. parvipinnis	A	1	1.8	4.20**	

† One (of 8) intact controls failed at a salinity of 1.3‰.

* Some failure occurred in intact controls after several months in fresh water.

** Of 3 intact controls, one failed at 4.1‰ and the others shortly after the salinity fell below 1.0‰.

Hypophysectomized brackish-water species tested by gradual dilution of their milieu failed at salinities between about 1‰ to 4‰ (Table III). Considerable variability in the salinity at failure was in evidence both within and between species. The former is especially true of *F. jenkinsi*, *F. luciae* and *F. zebrinus* where the omission of a single abnormally high value would substantially reduce the mean. We are hesitant to aribitarily exclude these values as failure in *F. heteroclitus* can occur in media iso- or even somewhat hyperosmotic to the body fluids (Burden, 1956; this report). With the exception of *F. parvipinnis*, intact controls generally survived well when subjected to gradually-decreased salinity. One (of 3) intact *F. parvipinnis* failed at 4.1‰ and the others failed shortly after the salinity fell below 1.0‰. A single (of 8) intact *F. grandis* failed at 1.3‰, and some delayed failure was observed in intact *F. majalis* and *F. luciae* after several months in fresh water. It is worth noting that when allowance is given for the time until failure following abrupt transfer and the additional delay in failure time in dilute sea water, failure tested by gradual dilution is consistent with an initiation of the process at salinities near presumed iso-osmotic levels for most brackish-water species.

Of the 14 freshwater species of *Fundulus* and one species of *Profundulus* in which freshwater tolerance was tested, none showed average times until failure of less than one month (Figure 1). Practical considerations prevented the continuation of most tests past a few months, but in the cases where this was possible (*F. rathbuni*, *F. notatus*, *F. olivaceus*, and *F. cingulatus*) the pattern of failure showed a gradual attrition as is also suggested by abbreviated tests on most other species. Even in specimens failing after very long periods of time (over one year in the case of some *F. notatus*) the symptoms accompanying failure (asthenia, extreme pallor, loss of balance) were identical to those in failing brackish-water species. The studies on *F. rathbuni* are indicative of high mortality occurring at about one month after transfer to fresh water. In this species a very high percentage (72%) of hypophysectomized specimens develop kidney stones after a few weeks which can occlude the kidney ducts and might be responsible for failure (Griffith and Pang, 1969). Such stones may also occur in a substantial percentage of hypophysectomized *F. heteroclitus* (Pickford, 1953), *F. diaphanus*, *F. waccamensis*, *F. catenatus*, *F. olivaceus* and in a few specimens of some other species. The relatively short survival of hypophysectomized *F. notti* and *Profundulus punctatus*, which were tested only at 20° C, might be extended somewhat for comparison with species tested only at 15° since survival at 20° appears to be shorter in most species of *Fundulus* tested at both temperatures.

A clear dichotomy exists between those species of *Fundulus* characteristic of brackish-water and those characteristics of freshwater environments in regard to survival in fresh water after hypophysectomy. The former fail within about a week or less and the latter survive for at least one month and generally much longer. *F. zebrinus*, an inland species which may frequent either freshwater or saline environments (Lewis, 1957) shows an intermediate condition. In this species environmental calcium plays a very important role in adapting to fresh water (Pickford, Pang, Stanley and Fleming, 1966). Within the brackish-water species the correlation between normal environmental salinity and survival in fresh water after hypophysectomy is still in evidence. Abbreviated survival is found in *F. parvipinnis*, *F. luciae* and *F. majalis*, the most halophilic species in nature (Griffith, 1974), while *F. jenkinsi* which survives the longest is found at low salinities. The relationship breaks down within the freshwater species. While freshwater species of *Fundulus* inhabit a variety of different environments including swamp water with very low levels of dissolved electrolytes (*F. cingulatus*, *F. swampinus*, *F. notti*), mineral-rich streams or lakes (*F. catenatus*, *F. sciadicus*, *F. seminolis*, *F.* sp.), intermediate conditions (*F. waccamensis*, *F. rathbuni*, *F. stellifer*), or a variety of different habitats (*F. chrysotus*, *F. notatus*, *F. olivaceus*, *F. diaphanus*) there is not even a suggestion that these habitat differences are reflected in survival time. *F. diaphanus*, which not infrequently enters low brackish water in estuaries, shows survival times very similar to those of its close relative *F. waccamensis*, a lake endemic.

There is little evidence that differences between species of *Fundulus* in freshwater tolerance after hypophysectomy are strongly correlated with evolutionary relationships within the genus. *F. notatus* and *F. olivaceus*, a closely related species pair, share very long survival times. On the other hand, what differences there are between survival in the other freshwater species are not suggestive of evolutionary stability of this feature. *F. catenatus*, *F. stellifer* and *F. rathbuni* form a close group phylogenetically, but *F. rathbuni* fails relatively rapidly and the others slowly. Nor is there any evidence for a close correlation between pituitary-dependent freshwater tolerance and various phylogenetic schemes proposd for the brackish-water species of *Fundulus* (*cf.* Brown, 1957; Miller, 1955; Farris, 1968; Chen; 1971; Relyea, 1967; Griffith, 1972). Since it is likely that freshwater species of *Fundulus* were derived from brackish-water ancestors by at least two independent invasions of fresh water (Griffith, 1972), and that freshwater adaptation in the genus *Profundulus* was probably independent of that in *Fundulus*, it would appear that the long survival of freshwater species of the genera *Fundulus* and *Profundulus* is principally determined by the environmental history of the species and that stability of the feature within phylogenetic lines plays a subsidiary role.

TABLE IV

Effect of hypophysectomy on serum electrolytes of F. heteroclitus *in various media at 20° C*

Group	Condition	Salinity	Time in medium	Health	Number	Serum electrolytes (values in mEq/1 ± S.E.)		
						Sodium	Potassium	Chloride
D1	Intact	SW	Long-term	Good	5	181.0 ± 3.8	7.8 ± 0.2	142.1 ± 3.2
D2	Hypect.	SW	Long-term	Good	5	175.3 ± 1.2	5.9 ± 0.3	124.2 ± 5.2*
C1	Mock-op.	FW	12 days	Good	5	179.2 ± 2.4	5.8 ± 0.3	124.8 ± 1.6
C2	Hypect.	FW	4-11 days	Failing	6	67.5 ± 3.6*	17.9 ± 4.9*	30.7 ± 3.3*
C3	Hypect.	1‰	8-16 days	Failing	3	103.3 ± 10.4†	15.0 ± 4.2	31.3 ± 3.7
C4	Hypect.	6‰	11-16 days	Failing	3	132.3 ± 12.7†	20.4 ± 6.9	58.3 ± 2.7†
C5	Hypect.	12‰	24-58 days	Failing	3	155.9 ± 13.1†	26.1 ± 6.3	138.0 ± 29.1†
C6	Mock-op.	FW + Ca^{++}	12 days	Good	4	183.0 ± 3.5	7.8 ± 0.9	132.8 ± 2.1†
C7	Hypect.	FW + Ca^{++}	5-9 days	Failing	6	77.4 ± 4.0*	11.8 ± 3.3	37.4 ± 2.8*

* Significantly different from intact or mock-operated controls in same medium, $P < 0.05$.

† Significantly different from comparable freshwater group, $P < 0.05$.

Because of the intolerance of most freshwater species of *Fundulus* for sea water (Griffith, 1974) and the poor survival of some brackish-water species in fresh water, it was generally not possible to use identical experimental methods on the two groups of fishes. Hence, we must at least consider the possibility that differences in method might account for the observed differences between the two groups in respect to freshwater tolerance after hypophysectomy. This would appear to be most unlikely from the fact that brackish-water species fail even when environmental salinity is reduced very gradually or when they are hypophysectomized in dilute sea water and then tested by transfer to fresh water as were the freshwater species. Burden (1956) tested *F. heteroclitus* hypophysectomized in fresh water and found little difference in failure time between such fish and those hypophysectomized in sea water and tested by abrupt transfer to fresh water. Mean failure time was 7.7 days for the former and 6.5 days for the latter, although a few freshwater-adapted fish held on for over two weeks.

Effect of hypophysectomy on serum electrolytes in five species of Fundulus

Data is given in Tables IV and V demonstrating the effect of hypophysectomy on serum electrolytes (sodium, chloride and potassium) in five species of *Fundulus.* In *F. heteroclitus* (Table IV), hyphophysectomy has only minor effects on blood electrolytes of seawater fish but results in profound changes following transfer to fresh water. In sea water (groups D1 and D2), there is a barely significant ($0.02 < P < 0.05$) depression of serum chloride but no change in serum sodium or potassium. Blood taken at the time of failure from hyphophysectomized fish in fresh water (group C2) showed a 75% decline in chloride, 62% decline in sodium and a 200% increase in potassium when compared with serum of mock-operated controls killed after 12 days in fresh water (group C1). A comparison of our data with that of Pickford, Griffith, Torretti, Hendler and Epstein (1970) on hypophysectomized fish autopsied after 3 days in fresh water, indicates that such fish have serum chloride and sodium levels intermediate between those of intact fish in fresh water and failing hypophysectomized fish in this medium. This suggests that the decline in serum electrolytes in *F. heteroclitus* following hypophysectomy in fresh water is a gradual process, although it must be noted that the study of Pickford *et al.* (1970) was conducted at 15° C and the data are not directly comparable with ours, which were obtained from 20° C fish.

Table V

Effect of hypophysectomy on serum electrolytes of four species of Fundulus *in fresh or sea water (15° C)*

Species	Group	Condition	Salinity	Time in medium	Health	Number	Serum electrolytes (values in mEq/l ± S.E.)		
							Sodium	Potassium	Chloride
F. majalis	E1	Intact	SW	Long-term	Good	7	175.1 ±2.9	11.3 ±1.8	163.0 ±2.4
	E2	Hypect.	SW	Long-term	Good	5	167.8 ±5.5	12.6 ±3.0	155.0 ±10.4
	C1	Intact	FW	8–49 hours	Failing	5	92.6 ±5.1	20.7 ±4.6	63.3 ±3.9
	C2	Hypect.	FW	21–30 hours	Failing	5	76.4 ±4.6*	35.3 ±4.3*	49.1 ±5.1*
F. diaphanus	A1	Intact	SW	7 days	Good	7	189.3 ±7.0	5.3 ±0.8	148.4 ±6.7
	A2	Hypect.	SW	7 days	Good	5	184.1 ±5.0	9.6 ±3.1	168.1 ±2.7*
	B1	Intact	FW	Long-term	Good	6	143.0 ±1.5	9.3 ±0.4	106.7 ±6.3
	B2	Hypect.	FW	8 days	Good	8	136.4 ±2.0*	11.9 ±1.3	104.8 ±3.3
	B3	Hypect.	FW	48 days	Good	6	111.6 ±3.6**	18.6 ±1.8	63.0 ±2.8**
	B4	Hypect.	FW	16–48 days	Failing	6	75.1 ±3.8**	24.7 ±0.7*	39.8 ±2.0**
F. rathbuni	B1	Intact	FW	Long-term	Good	4†	—	—	112.4 ±1.2
	B2	Hypect.	FW	29 days	Good	6	—	—	84.1 ±3.1*
	B3	Hypect.	FW	12–200 days	Failing	11	—	—	58.8 ±5.3**
F. swampinus	B1	Intact	FW	Long-term	Good	5	—	—	97.8 ±3.4
	B2	Hypect.	FW	31 days	Good	7	—	—	88.9 ±4.1

* Significantly different from intact controls, $P < 0.05$.

** Significantly different from both intact controls and preceding hypect. group, $P < 0.05$.

† Pooled samples, two fish per sample.

In *F. majalis* (Table V) we found no significant effect of hypophysectomy on blood electrolytes in sea water, although there is a trend towards slightly lower sodium and chloride levels. Both intact and hypophysectomized *F. majalis* are unable to tolerate abrupt transfer from sea to fresh water and fail at the same time (22.4 ± 7.5 hours for the intacts and 23.9 ± 1.6 hours for hypophysectomized fish). Nevertheless, intacts at failure have significantly higher serum sodium and chloride and significantly lower potassium than comparable hypophysectomized fish.

Hypophysectomized *F. diaphanus* in sea water have barely significantly higher serum chloride than intacts but other blood electrolytes are close to control levels. In fresh water there clearly are progressive declines with time of serum chloride and sodium and a concomitant increase in potassium in hypophysectomized fish. At the time of failure serum chloride values are 38%, sodium levels 52%, and potassium levels 265% the values of intact controls in fresh water.

Only serum chloride levels were measured for *F. rathbuni* and *F. swampinus*. Both species survive for fairly long periods in fresh water following hypophysectomy. In *F. rathbuni* sacrificed after 29 days in fresh water, hypophysectomized fish show significantly lower serum chloride when compared with intact controls. Serum chloride is further depressed in hypophysectomized fish showing the symptoms of failure. In *F. swampinus* there is a suggestion of lower chloride levels in hypophysectomized fish sampled after 31 days in fresh water, although the difference is not significant ($0.1 < P < 0.2$).

Several features in common, as well as a number of differences, are evident when a comparison is made of the responses of the species of *Fundulus* to hypophysectomy In the three species studied, serum electrolyte changes after hypophysectomy in sea water are minor, involving slight decreases in serum chloride in *F. heteroclitus* and slight increases in *F. diaphanus*. Changes after hypophysectomy in seawater-adapted *F. heteroclitus* were documented in much more detail by Srivastava and Pickford (1972) who found significant decreases in serum sodium and chloride as well as changes in many other blood constituents. Our data confirm the chloride data and we also note a trend towards lower sodium in *F. heteroclitus* and similar trends for both sodium and chloride in *F. majalis*. The fact that *F. diaphanus* exhibits the opposite response with chloride rising rather than falling after hypophysectomy, may reflect species differences influenced by habitat between this normally freshwater species and the brackish-water *F. heteroclitus* and *F. majalis*.

In fresh water it is clear that hypophysectomy results in profound changes in blood electrolytes in at least four of the five species tested which evenutally lead to extremely low values for serum sodium and chloride and high potassium. With the possible exception of *F. rathbuni*, the levels of serum ions at the time of failure are not significantly different between species, suggesting that tissue tolerance to lowered blood electrolyte levels is essentially identical in *F. majalis, F. heteroclitus* and *F. diaphanus*. Some failing *F. rathbuni* have relatively high serum chloride levels (up to 78 mEq/l), while others are in the "normal" range for failing fish of the other species (31–38 mEq/l). It is possible that the former were failing from causes other than depressed blood electrolyte levels. It is worth noting that the levels of serum chloride we find in failing *Fundulus* species are lower than those reported by Burden (1956), but that levels of both sodium and chloride are similar to those reported to be consistent with failure in the goldfish, *Carassius auratus*, by Lahlou and Sawyer (1969). It is possible that Burden sampled fish earlier in failure than we did. The quantitatively smaller depression of serum osmolality reported by Pickford, Pang and Sawyer (1966) probably reflects increases in serum constituents such as potassium in failing fish.

In *F. diaphanus* our data show that the decline in serum sodium and chloride are progressive and this is also strongly suggested by the data on *F. rathbuni* and *F. heteroclitus.* We have no reason to suspect this not to be the case in other species of *Fundulus*, and it seems reasonable that differences between species in tolerance of fresh water after hypophysectomy are the result of differences in the rate of decline of serum electrolytes. In *F. majalis* the decline in serum electrolytes is very rapid following transfer to fresh water and results in levels low enough to account for mortality within one day. The failure of intact as well as hypophysectomized fish suggests that hypophysial control (which is present as can be seen from the significant difference in serum electrolytes in failing fish and the ability of intacts to survive in fresh water following gradual acclimation) is inadequate to compensate for the rapid decline in electrolytes following abrupt transfer to fresh water. In *F. heteroclitus* the decline is somewhat slower and levels comparable to those of failing *F. majalis* do not occur until several days after transfer. Declines in serum electrolytes are yet more retarded in the freshwater species and levels low enough to be considered responsible for failure do not normally occur until a month or more after transfer from dilute sea water to fresh water.

While it is probably a fair assumption that the declines in serum electrolytes observed here are the result of imbalances between the influx of ions across the gills and the renal and extrarenal losses of electrolytes, it has recently been suggested (Duff and Fleming, 1972a, 1972b) that modifications in "sodium space" (*i.e.*, a redistribution of sodium to other body compartments) can provide a mechanism by which some species of *Fundulus* may regulate serum sodium at relatively constant levels. In contrast, Lahlou and Sawyer (1969) have reported that hypophysectomized goldfish, *Carassius auratus*, show marked declines in serum electrolytes while 'sodium space' increases, resulting in only minor changes in the pool of exchangeable sodium. Although our data do not permit us to distinguish whether differences in survival after hypophysectomy in fresh water between brackish-water and freshwater species of *Fundulus* are attributable to sodium flux rates or the capacity to modify "sodium space," the more pertinent observation is that, whatever their cause, these differences exist.

Effects of salinity and calcium on serum electrolytes of failing hypophysectomized F. heteroclitus

Data is presented in Table IV demonstrating the effect which elevated environmental salinity and calcium levels have on serum electrolytes of failing hypophysectomized *Fundulus heteroclitus.* With an increase in salinity there is a parallel increase in serum chloride and sodium of failing fish (Groups C2–C5). Specimens failing at 12‰ have serum chloride and sodium levels approaching those of intact specimens in fresh water or healthy hypophysectomized fish in sea water. It would appear that under the exceptional conditions of failure in moderately high salinities, the serum electrolytes measured are not closely related to failure. If lowered serum chloride and sodium are not causing failure in dilute sea water, it is worth considering what factors might be responsible. One possibility, suggested by the fact that high sodium levels in fish failing in dilute sea water are accompanied by very high potassium levels, is that the ratio of potassium to sodium is the critical factor. However, a comparison of sodium and potassium levels for individual failing fish indicated that this was unlikely. The correlation coefficient for sodium and potassium levels of all failing *F. heteroclitus* was 0.178 ($0.4 < P < 0.5$). A second possibility is that failure in both fresh water and dilute sea water and low serum electrolyte levels in fresh water are all consequences of a common phenomenon; perhaps a disturbance in acid-base metabolism. Of special interest in this regard is the observation of Srivastava and Pickford (1972) that hypophysectomy results in elevated bicarbonate levels in seawater-adapted *F. heteroclitus* and the evidence, summarized by Maren (1967) and Maetz (1971), that sodium and chloride uptake and acid-base metabolism share a common pathway through the carbonic anhydrase system.

High levels of environmental calcium are without effect on the serum chloride, sodium and potassum levels of failing hypophysectomized *F. heteroclitus* in fresh water (groups C2 and C7, Table IV). High environmental calcium levels enable many marine fishes to adapt to fresh water (Breder, 1934; Hulet, Massay, Joorey and Wehr, 1967), reduce chloride loss in *Gasterosteus aculeatus* (Heuts, 1944), and decrease water and sodium fluxes in *Fundulus zebrinus* (Potts and Fleming, 1970, 1971). Calcium deprivation is without effect on serum sodium, chloride or potassium in hypophysectomized *F. heteroclitus* in sea water (Pang, Griffith and Pickford, 1971) or hypophysectomized *F. diaphanus* in fresh water (Pang, Griffith and Schreibman, 1973), although such fish demonstrate marked declines in serum calcium and exhibit tetanic seizures.

Discussion

Our findings point to the likelihood that failure in fresh water after hypophysectomy is qualitatively comparable between different species of *Fundulus*. In all species tested, the symptoms of failure (extreme pallor, sluggishness, loss of balance and evenutal death) are identical. With the notable exception of the failure of *F. heteroclitus* in dilute sea water, failure in both freshwater and brackish-water species is closely correlated with very low serum sodium and chloride. The levels of these ions accompanying failure appear to be similar for both groups of species.

In contrast to the qualitative similarities, there are marked quantitative differences between species of *Fundulus* in freshwater failure after hypophysectomy which appear to be related to normal environmental salinity. The exact mechanisms by which these quantitative differences arise (*i.e.*, by reduced renal and/or extrarenal electrolyte efflux, by involvement of environmental calcium in electrolyte and water balance, by increased ion uptake, or by modification of electrolyte "spaces" in the late-failing freshwater species) have not yet been clarified and represent a rich source of problems for future investigation. However, what seems to us more pertinent here than the nature of the mechanisms by which freshwater species of *Fundulus* achieve longer survival after hypophysectomy than their brackish-water congeners is the very fact that, at least from the quantitative index of survival time, in these freshwater species the role of the pituitary is of less importance in adapting to fresh water.

It is our contention that a strong reliance on endocrine control of osmoregulation, at least in *Fundulus*, is most critical in euryhaline species which are subject in nature to variations in salinity from freshwater to marine (or higher) levels.

The principal known actions of prolactin on osmotic and ionic regulation (*cf.* reviews by Lam, 1972; Ensor and Ball, 1972) are all mechanisms by which the osmoregulatory physiology of a marine teleost can be transformed into that of a freshwater teleost (*cf.* Smith, 1930). It might be expected that strictly freshwater species, lacking any need for mechanisms of regulating ion and water balance in a hypertonic medium, would eliminate any such vestiges of marine life through evolutionary rather than through endocrine pathways, so that a metabolically-costly continuous secretion of hormones would not be necessary to survival. Euryhaline species would need both the capability of osmoregulating like a marine teleost in saline environments and an effective means, presumably hormonal, or modifying the mechanisms involved for adaptation to fresh water. While there are no stenohaline marine species of *Fundulus*, our data on the more halophilic species such as *F. majalis* suggest, and it is intuitively reasonable, that forms which have minimal exposure in nature to dilute environments neither need, nor possess, well-developed hypophysial control of osmoregulation.

Of considerable interest is the question of whether our data on *Fundulus* indicative of a close relationship between environment and the importance of the pituitary in osmoregulation, are representative of a widespread phenomenon in fishes or are only of restricted applicability to *Fundulus*. The only other series of studies of sufficient taxonomic breadth to be helpful in deciding this point are those of Schreibman and Kallman (1966, 1969) on poeciliid fishes. These authors found that species of the exclusively freshwater genus *Xiphophorus* demonstrate relatively long survival while the genus *Poecilia*, which includes a number of brackish-water species, has short survival; the shortest survival being found in the highly euryhaline *P. latipinna* and *P. formosa*. However, Schreibman and Kallman (1969) reported that *Gambusia affinis*, presumed to be ecologically similar to *Poecilia latipinna* on the basis of a report by Krumholz (1948), has relatively long survival and concluded that there was no consistent relationship between environmental salinity and survival after hypophysectomy in fresh water. An examination of more recent extensive field surveys (Kilby, 1955; Simpson and Gunter, 1956; Renfro, 1960; Tagatz and Dudley, 1961; Tagatz, 1968) reveals, however, that *G. affinis* is normally an inhabitant of fresh water and only sporadically enters low brackish environments, while *P. latipinna* is fully euryhaline in nature. Thus, the poeciliid data of Schreibman and Kallman (1966; 1969) exhibit a rather good correlation between environmental salinity and freshwater survival after hypophysectomy.

Generalizations for teleosts as a whole are complicated both by the scattered nature of the available data on most groups and the widely varying evolutionary and environmental history of different teleost taxa. Schreibman and Kallman (1966; 1969), in summarizing the studies on teleosts, concluded that certain taxonomic groups (*i.e.*, the ostariophysian, anguillid and perhaps salmonid fishes) appeared not to require the pituitary for survival in fresh water while the atheriniform fishes (which include *Fundulus*, the poeciliids, and a number of other groups, only a few of which have been tested) did. These authors further suggested that within teleosts as a whole there was no consistent relationship between environmental salinity and pituitary-dependent freshwater tolerance. Some doubt as to the validity of the suggested taxonomic restriction of the hypophysial mechanism for adapting to fresh water is raised by observations that some ostariophysian and salmoniform fishes require the pituitary to survive in fresh water (Chidambaram *et al.*, 1972; Stanley and O'Connell, 1970), other ostariophysian and some anguillid fishes need the pituitary for maintenance of serum electrolytes (Ogawa, 1968; Lahlou and Sawyer, 1969; Donaldson, Yamazaki and Clarke, 1968; Olivereau and Chartier-Baraduc, 1966; Chan, Chester Jones and Mosley, 1968; Butler, 1967), and our data on *Fundulus* (an atheriniform genus) represent both the shortest (about one day in *F. luciae* and *F. majalis*) and longest (up to a year or more in some *F. olivaceus* and *F. notatus*) pituitary-dependent survival in fresh water reported for teleosts. While we cannot deny the likelihood that phylogenetic differences occur in reliance on the pituitary for freshwater adaptation, present data suggest strongly that the phenomenon is widespread, if not ubiquitous, in freshwater and euryhaline teleosts and that it broadly crosses taxonomic boundaries. We suspect that the mechanism, involving prolactin in every case yet tested, is a primitive feature of teleosts and that differences between taxa in the importance of the mechanism are principally a function of the environmental history of the group.

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SUMMARY

1. Eight species and one subspecies of *Fundulus* native to brackish-water environments fail within 1–10 days following abrupt transfer to fresh water and fail between 1–4‰ when subjected to gradual declines in salinity following hypophysectomy. Intact specimens of most species survive indefinitely in fresh water.

2. Of fourteen species of *Fundulus* and one species of *Profundulus* typical of freshwater habitats, none fail before one month and some survive for periods of up to one year following hypophysectomy and transfer to fresh water.

3. One inland species of *Fundulus* characteristic of saline and freshwater environments demonstrates a failure pattern intermediate between the brackish-water and fresh water species of the genus.

4. In those species tested at two or more different temperatures, low temperature generally prolongs survival in fresh water after hypophysectomy.

5. Although hypophysectomy has only minor effects on serum electrolytes (sodium and chloride) in the three species of *Fundulus* tested in sea water, it

results in marked declines in serum sodium and chloride in fresh water in the five species tested. Extremely low serum electrolytes are associated with failure: the levels at failure do not differ between species but the rate of decline is much slower in freshwater species than in brackish-water forms.

6. Hypophysectomized *F. heteroclitus* fail even at salinities iso-osmotic with serum (12‰), although survival in dilute sea water is much prolonged over that in fresh water and fish survive indefinitely in sea water (29‰). Failure in dilute sea water is not associated with very low serum chloride and sodium, suggesting that factors other than the regulation of these electrolytes may be involved in the process of failure.

7. We conclude that the relative importance of the pituitary in adapting to fresh water is closely correlated with normal environmental salinity in the genus *Fundulus;* the hypophysis being most critical to those species which are subjected to both marine and freshwater conditions in nature.

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THE CONTROL OF EGG MATURATION BY JUVENILE HORMONE IN THE TOBACCO HORNWORM MOTH, *MANDUCA SEXTA*

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In a variety of insect species, the adult female requires the presence of active corpora allata (CA) for normal ovarian development. The CA, at this stage, secrete juvenile hormone (JH) which is, presumably, the same compound(s) responsible for the maintenance of larval characters during the early development of the individual (Wigglesworth, 1948; Williams, 1961; deWilde and deLoof, 1973). In *Manduca sexta,* Judy, Schooley, Dunham, Hall, Bergot, and Siddall (1973) have found at least one JH which is secreted by the adult female CA *in vitro* and which can be extracted from larval blood. In some species, JH appears to control the synthesis of the female specific yolk protein (see Engelmann, 1970; Wigglesworth, 1970; Wyatt, 1972; Doane, 1973; deWilde and deLoof, 1973 for reviews). But this is by no means a general rule. Those species which have been shown to have a vitellogenin protein not controlled by JH belong to the Saturniidae (Pan and Wyatt, 1971; Telfer, 1965) and the Culicidae (Hagedorn and Fallon, 1973).

The action of JH in ovarian development in adult female insects is not limited to control of yolk protein synthesis. In some cockroaches, oocytes will not incorporate vitellogenin unless JH is present (Bell, 1969; Bell and Barth, 1971). In *Aedes aegypti,* although synthesis of vitellogenin is not controlled by JH, the CA are required for egg maturation (Lea, 1963, 1967). In this instance, JH acts to promote development of very young follicles to the normal resting stage (Gwadz and Spielman, 1973).

Sroka and Gilbert (1971) first reported that JH was necessary for egg development in the adult female sphinx moth, *Manduca sexta*. Our study was concerned with the role played by JH in this ovarian maturation. The evidence points to a new type of gonadotropic action of JH that is not concerned with either vitellogenin synthesis nor its uptake. Rather JH seems to be permissive in allowing oocytes to mature beyond the vitellogenic phase.

MATERIALS AND METHODS

Experimental animals

The rearing procedure for larval tobacco hornworms has been described (Truman, 1972). Larvae were raised at 25° C in a long day photoperiod (17L:7D). Developing adults were stored in polyethylene bags and were switched to a short day photoperiod (12L:12D) at 25° C, two weeks after pupation. Adult eclosion is more synchronous under the 12L:12D photoperiod (J. W. Truman, M. M. Nijhout, personal observations). Pharate adults were checked each hour during the eclosion gate (Truman, 1971), and emerged animals were separated and held in groups of five or fewer in paper bags during the course of the experiment. They were not fed nor allowed to mate.

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Surgical methods

Gland extirpations from pupae were performed following the classical techniques perfected by Williams (1959) on the Cecropia silkmoth pupa, except that no Ringers solution was added to the animal after surgery. Glands were removed to a black watch glass and examined with a dissecting microscope to verify complete removal. A few crystals of 1 : 1 mixture of phenylthiourea and streptomycin sulfate were placed in all wounds. Animals from which the brain had been removed within 24 hours of the pupal ecdysis were injected 10 days later with 8 μg of β-ecdysone dissolved in 50 μl of 10% isopropanol.

Gland extirpations from adult moths were performed approximately 4 hours after adult eclosion. Williams' (1946) technique of continuous CO_2 anesthesia was used, the moth being suspended in the Buchner funnel by placing the neck region in a slit of cardboard which lay across the mouth of the funnel. An incision was made just posterior to the head on the dorsal neck and the CA removed with fine forceps to a black watch glass. Usually, the glands remained attached to a piece of gut or heart. The wound was filled with a piece of Gelfoam (Upjohn Co.).

To remove glands from an adult for subsequent implantation, the head was isolated and all its appendages and the compound eyes removed. Further operations were done with the head submerged in saline. The dorsal surface of the head can then be removed by making two shallow cuts through the cuticle anterior and posterior to the antennal sockets and teasing the cuticle away from the muscles. Removing the remaining musculature and tracheal system exposes the brain and the corpora allata and corpora cardiaca (CC). Appropriate transections with microscissors allows one to remove this entire complex to a black watch glass for observation with a dissecting microscope. The glands which were attached to a piece of heart were removed from the brain. The CA were separated from the CC by one cut. The piece of heart was left attached for easy manipulation of the glands. Implants into adults were made through a lateral incision between the first and second abdominal segment and the wound filled with Gelfoam.

Juvenile hormones

The juvenile hormones or JH mimics were dissolved in light mineral oil (Fisher, Saybolt viscosity 125/135). Fifty microliters of these solutions were injected into the first abdominal segment of the anaesthetized adult using a 26 gauge needle on a disposable 1 ml syringe (Plastipak) controlled by an Agla micrometer. The compounds used in this study were the synthetic Cecropia juvenile hormones, C18-JH (Zoecon Corp., Insect Control) and C17-JH (Insect Control); C16-JH (Insect Control); ethyl 3,7,11-trimethyldodeca-2,4 dienoate (ZR512, Zoecon Corp.); and farnesyl methyl ether (A. M. Ajami).

Dissections

The extent of development of the ovary, in most cases, was determined by the number of mature, fully chorionated eggs present. The abdominal contents of a moth were removed to absorbent paper and the viscera spread with moderate pressure of the finger. Fully mature eggs resisted pressure whereas immature follicles and fat body were disrupted and absorbed into the paper. The mature eggs could then be counted. Unless otherwise noted, dissections of this type were performed 4 days after adult eclosion.

For observation single ovarioles were dissected out under a modification of a saline developed for *Manduca* (Cherbas, 1973). They were placed in this saline in a black watch glass and observed with a dissecting microscope.

SDS gel electrophoresis

The disc-gel electrophoresis procedures of Davis (1964) were modified and used as an SDS stacking gel system by adding 0.1% SDS (final concentration) to all buffers as suggested by Weber, Pringle, and Osborn (1972). Lower gels were 6% acrylamide and were cast in tubes 5 mm ID. They were 7 cm long. The upper stacking gel was 3% acrylamide, 0.029 M Tris-phosphate, 0.1% SDS and was 200 μl in volume. Samples were added in 50 or 100 μl of sample buffer containing 1% SDS, 0.024 M Tris-phosphate, 10% glycerol by volume, 0.01 M 2-mercaptoethanol, 0.005% bromphenol blue. The electrolyte buffer was 0.01 M Tris, 0.065 M glycine, 0.1% SDS. Electrophoresis was performed at room temperature with a current of 2.5 mAmp per tube for 90 minutes. Gels were stained in 0.01% Coomassie Brilliant Blue (Sigma) for 24 hours and destained for the same length of time. They were scanned with a Gilford 2400 recording spectrophotometer. Percentage of total protein detectable by this method was estimated by weighing cut-outs of the absorbance tracings.

Samples consisted of one μl of hemolymph collected from a cut in the wing of an adult animal as it inflated its wings just after eclosion or by slow centrifugation of older animals with their appendages amputated. Blood was taken from new pupae through cuts in the proboscis and from pharate adults through incisions in the dorsal abdomen. PTU was added to inhibit tyrosinase activity. Blood samples were spun for 2 minutes in a Beckman microfuge. Egg samples were prepared by freezing, thawing, and pipetting mature or immature follicles in a buffer containing 0.024 M Tris-phosphate (pH 6.9) and 1% SDS. The equivalent of one half egg or follicle was added to the gel. All samples were heated in sample buffer at 100° C for 2 minutes.

RESULTS

Normal morphology of ovarian development in the adult

At the time of adult eclosion, a female *Manduca* has no mature eggs in its ovaries. The oldest follicles are 1 mm in length, contain yolk in the oocyte and retain a nurse cell cap (see Figure 1a). During the next 24 hours, these follicles will complete vitellogenesis and the terminal phase of oogenesis (Telfer and Anderson, 1968) including hydration and chorion synthesis (see Figure 1b). The first mature eggs are found in the ovaries 24 hours after eclosion, by 4 days after eclosion, unfed virgin females contain, on the average, 100 mature eggs.

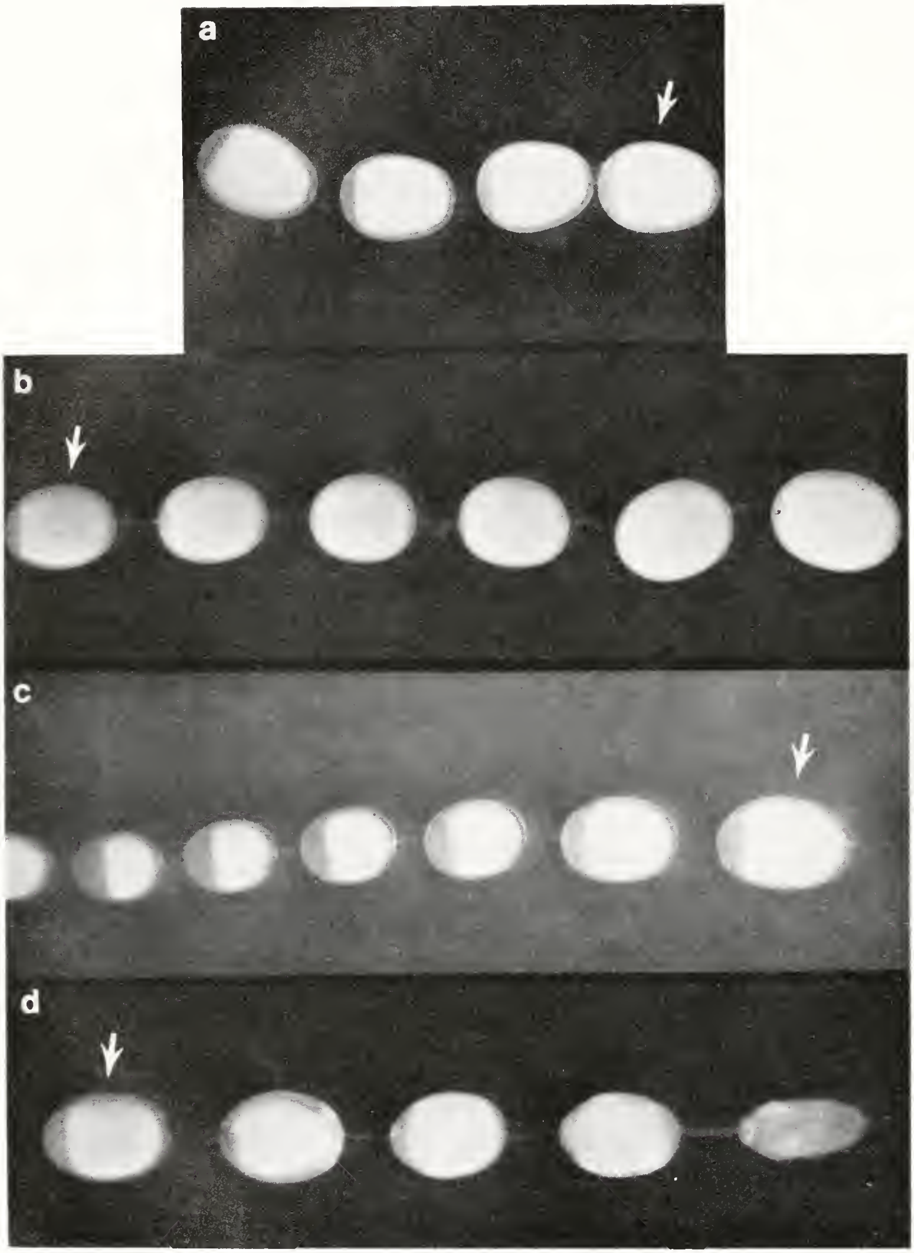

Figure 1. Ovarioles of intact and allatectomized females; the anterior end is to the left in all photographs, (a) intact female immediately after eclosion, (b) intact female 12–18 hours after eclosion. Note normal sequence of events beginning with arrow, (c) allatectomized female immediately after eclosion. Follicles appear normal, (d) allatectomized female 48 hours after eclosion. Note abnormal and degenerating follicles immediately posterior to the critical stage (arrow). In all cases arrows indicate follicles at the critical stage.

Effect of allatectomy on egg maturation

Female moths which had been allatectomized as pupae were held for 4 days after eclosion. Upon dissection, no mature eggs were found (Table I) as compared with sham-operated controls which matured almost the normal complement. When the operation was delayed until 4 hours after adult emergence, females were capable of making a small number of eggs in 4 days whereas sham-operated controls produced the normal number.

Although the ovarioles of females allatectomized as pupae appeared normal at the time of eclosion (Figure 1c), they showed widespread degeneration of the posterior follicles at the time of dissection or even within 24 hours after emergence (Figure 1d). In animals deprived of their CA for any length of time as adults, the immature follicles appeared normal until they reached a length of approximately 1 mm, the same stage of development found in the terminal position of the ovariole in newly emerged adults. The follicles posterior to this point appeared abnormal with discontinuities in the yolk and disruptions of the nurse cells. Eventually each follicle degenerated.

TABLE I

Effect of allatectomy on egg maturation in Manduca sexta

Operation	Number animals	Average number mature eggs (± S.E.M.)
No treatment	50	100 ± 6
Allatectomized as pupa	15	0
Sham operated as pupa	10	83 ± 10
Allatectomized as adult	10	7 ± 3
Sham operated as adult	5	97 ± 12

These results support those of Sroka and Gilbert (1971) who showed that the CA were necessary for egg maturation in *Manduca sexta.* Further, they indicate that the CA do not become active until after adult eclosion. In order to determine the time of onset of activity in the glands, a means of allatectomizing adults was needed which was not so tedious as the surgical procedure.

Newly emerged adults were anesthetized in CO_2 for 1–2 minutes. The animals were decapitated and the wound sealed with melted paraffin. As seen in Table II, this decapitation drastically reduced the ability to mature eggs. The ovarioles contained degenerating follicles, similar to those seen in allatectomized animals.

Removal of the head eliminates three potential endocrine sources: the brain, CC, and CA. To establish the relative importance of these organs, animals decapitated immediately after eclosion received implants of glands 12 hours after emergence. Twelve-hour old females donated either a brain, a CC-CA complex, or an isolated pair of CA. As shown in Table II, only implanted CA restored the ability to form completely mature eggs. (The presence or absence of the CC with the CA made no difference in the response so the results have been combined in the table). Further studies showed that adult male CA were relatively inactive in stimulating egg maturation whereas glands from mid-fifth instar larvae were as active as adult female glands.

TABLE II

Requirement for active CA for egg maturation in decapitated female moths

Treatment after decapitation	Number animals	Average number mature eggs (± S.E.M.)
None	50	1 ± 0.7
Implant adult ♀ brain	14	5 + 3
Implant adult ♀ CA (1 pr)	20	55 ± 7
Implant adult ♂ CA (1 pr)	5	13 ± 5
Implant larval CA (1 pr)	9	56 ± 17

The implanted CA restored the ability to form only half the normal egg complement in 4 days. Reasons for this will be discussed below.

Dose response curve of JH-induced egg maturation

A series of juvenile hormones and JH mimics were evaluated for their activity in promoting egg maturation in *Manduca.* The animals used for this assay were decapitated within one hour of emergence. The hormone solution was injected 12 hours later and the eggs counted on the fourth day. Only mature, fully chorionated eggs were counted.

Figure 2 shows that the number of eggs matured by decapitated animals depended on the dose of hormone they received. C18-JH and C17-JH were the most active compounds with 1 μg producing three times the control number of eggs in 4 days. C16-JH, a hormone isolated from adult female *Manduca* CA (Judy, *et al.*, 1973), was about 500 times less active, as was the ZR512 mimic. Farnesyl methyl ether, a relatively inactive JH mimic on *Manduca*, was no more

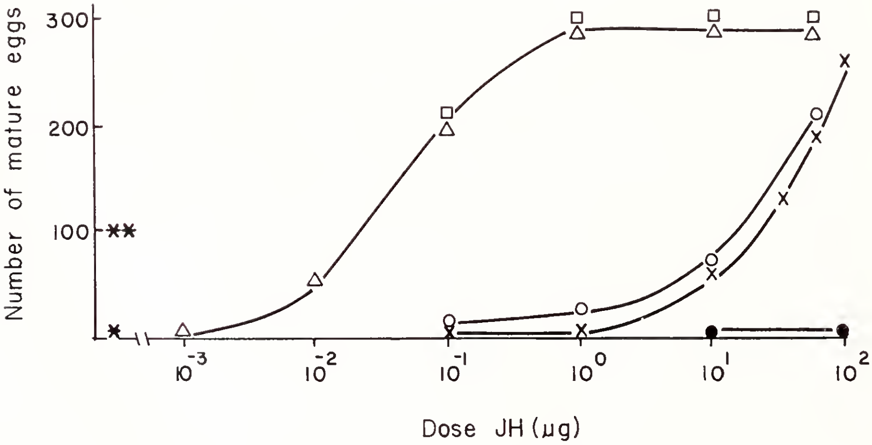

FIGURE 2. Dose-response curve of JH-induced egg maturation. Each point represents the average number of eggs matured in 4 days by 10 animals. Symbols used are: single asterisks, Oil injected controls; double asterisks, untreated females; squares, C17-JH: triangles, C18-JH; open circles, C16-JH; X-ZR512; closed circles, farnesyl methyl ether.

active at 100 μg than pure mineral oil. The order of activity compares favorably with that found in other assays developed on *Manduca* (Truman, Riddiford, and Safranek, 1973; Riddiford and Ajami, 1973).

Effect of brain extirpation on egg maturation

Sroka and Gilbert (1971) reported that removal of the brain from the female pupa resulted in the inability of the subsequent adult to develop eggs, as measured by increase in weight of the ovaries. We removed brains from each of 10 animals within 24 hours of pupation, then injected ecdysone to provoke adult development. All 10 females emerged but none survived for 4 days. Upon dissection they were found to contain no mature eggs, but also showed a curious lack of fat body. In contrast to decapitated animals, these brainless adults were hyperactive. Thus, the subesophageal ganglion without inhibitory influences from the brain seems to cause increased activity in *Manduca,* as Roeder (1935) had previously shown for the mantid, *Mantis religiosa.* This hyperactivity of brainless adults of *Manduca* had apparently caused a quick depletion of their fat body reserves. It was unclear whether they had not developed eggs because of a lack of hormonal stimulus or a lack of nutrient reserves.

To overcome this problem, a second experiment was performed in which the emerging brainless animals were injected with 5 μg of tetrodotoxin (TTX) in 50 μl distilled water. Paralysis by TTX prevented the rapid depletion of the reserves; when the animals were dissected 4 days later, the fat body appeared normal and healthy. None of these animals contained mature eggs (Table III). The morphology of the ovarioles was identical to that of allatectomized or decapitated females in that it showed the same terminal degeneration of follicles. The last normal appearing follicle was 1 mm long and contained yolk and a nurse cell cap. Autopsies showed that at least one CA was present in all animals. TTX-injected controls matured normal numbers of eggs.

Because decapitated animals responded so well to exogenous JH, we thought it unlikely that the brain was providing a second gonadotropic factor. But the possibility remained that the brain secreted a substance before eclosion which was

TABLE III

Effect of brain extirpation and JH injection on egg maturation

Treatment as pupa	Treatment as adult	Number animals	Average number mature eggs (± S.E.M.)
Sham operated	None	10	83 ± 10
Brain removed	None	10	0
Brain removed	5 μg TTX	7	0
None	5 μg TTX	8	125 ± 11
Brain removed	5 μg TTX and 50 μl pure mineral oil	4	0
Brain removed	5 μg TTX and 0.1 μg C17-JH	5	198 ± 16
None	Decapitated and 0.1 μg C17-JH	10	208 ± 20
Brain removed	5 μg TTX and 10 μg ZR512	5	90 ± 9
None	Decapitated and 10 μg ZR512	10	65 ± 7

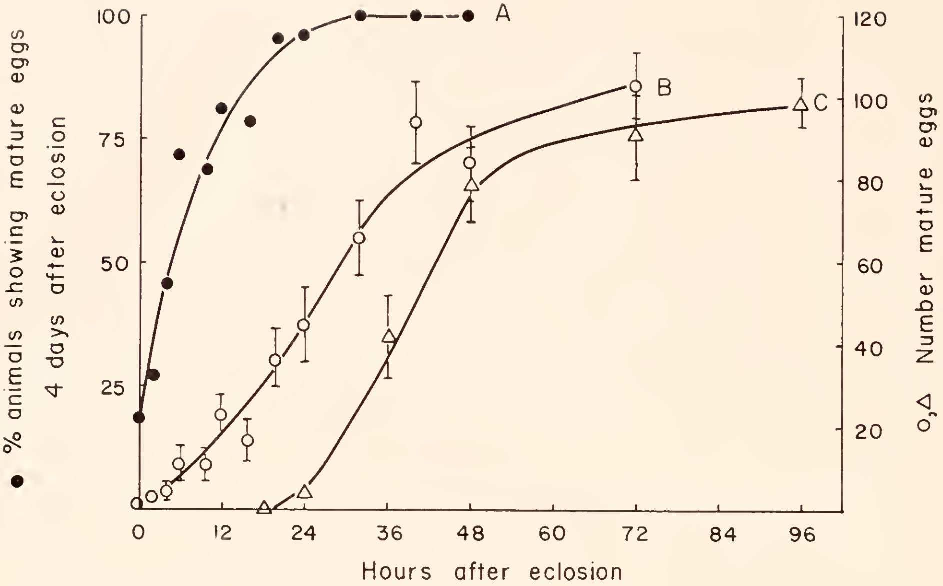

Figure 3. Activity of the CA after adult eclosion. Each point represents observations on 51 ± 3 animals except that at 18 hours which represents 20 animals. (A) per cent of animals which have activated CA by time of decapitation at various times after eclosion; (B) number of eggs matured by these decapitated females by 96 hours ± S.E.M.; (C) number of mature eggs present in intact females at various times after eclosion ± S.E.M.

necessary for ovarian development. Therefore, brainless female moths were used as assay subjects for JH. As soon as the brainless animal emerged, it received an injection of 5 μg TTX. Twelve hours later, 50 μl of a JH solution was injected; then the animal was dissected at 96 hours. Controls received 50 μl of pure mineral oil. Table III shows that individuals deprived of their brains throughout adult development responded as well to injected JH as did females decapitated after eclosion.

Activity of the CA in adult females

In order to study the activation of the CA after eclosion and the kinetics of JH release within the population, large numbers of animals were held for various periods of time after emergence and then decapitated. All were dissected on the fourth day after eclosion and the number of mature eggs counted. The results are shown in Figure 3.

Curve A shows that the ability of females to form mature eggs increased when decapitation was performed progressively later in adult life. At the time of emergence, 19% of the population was competent to mature a few eggs (average of 9 eggs) without the continued presence of the CA. Therefore, only a small amount of hormone had been released up to that time since this is only 9% of the normal complement of mature eggs. By 4 hours, 50% of the population had active glands. Decapitation at 32 hours no longer prevented egg maturation in any animal.

The fact that the number of eggs matured was dependent on the amount of JH injected into decapitated females as shown in Figure 2, suggested that the time course of JH release could be estimated by correlating the number of eggs produced in this same set of animals with the time of decapitation. Curve B in Figure 3 represents the rate of JH release. For instance, by 24 hours, enough hormone had been released into the moth's bloodstream to promote the maturation of 45 eggs whereas 12 hours earlier, a moth could mature only 24 eggs when deprived of its source of JH.

Curve C in Figure 3 is a reference curve showing the rate of appearance of mature eggs in intact females. The data for this curve were obtained by dissecting female moths at various times after eclosion and counting the mature eggs present in the ovaries.

The latter two curves show a horizontal displacement of 12 hours at the half-maximum point of 50 eggs. For example, an animal decapitated at 24 hours and dissected at 96 hours contains approximately the number of mature eggs characteristic of a 36 hour old female. This 12 hour period most likely represents the maximum time it takes for a follicle to complete development after becoming

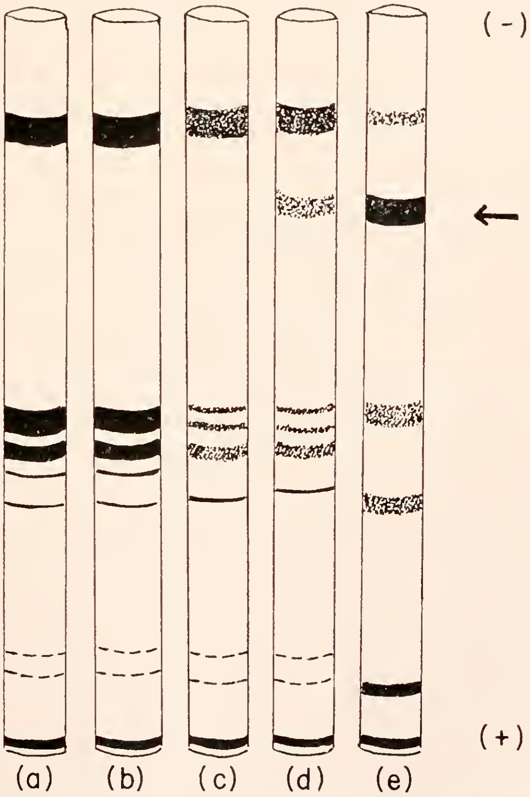

FIGURE 4. Electrophoretograms of hemolymph (a–d) and mature egg homogenate (e): (a) male pupa; (b) female pupa; (c) male adult; (d) female adult; (e) egg homogenate. Arrow indicates vitellogenin.

independent of JH or of the JH-controlled process. It is probable that the period of JH-independence is actually shorter since a finite time is required for the decline of the blood titer of JH.

Effect of allatectomy on vitellogenin concentration

In view of the fact that several insect ovarian maturation systems require JH for synthesis of the female-specific yolk protein, it was of interest to establish the presence of such a vitellogenin in *Manduca* and to determine the effect of allatectomy on its concentration in the blood and uptake into follicles.

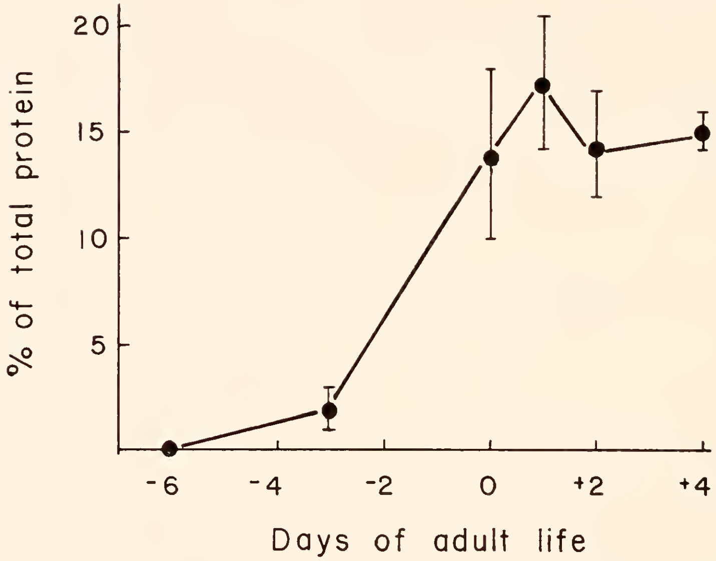

FIGURE 5. Concentration of vitellogenin in blood during development. Each point represents the mean value of 5 samples. Error bars show standard deviation.

When blood samples of early pupae and freshly-emerged adults were subjected to SDS gel electrophoresis, a sex-specific protein band was found in the adult female (see Figure 4). The same band was found to be a major protein component of the mature egg. This is, presumably, the vitellogenin protein.

In order to determine the time in development when the vitellogenin first appeared in the blood, hemolymph samples were withdrawn from developing adults at 3 day intervals after pupal ecdysis. No vitellogenin was found in animals immediately after pupation nor during early adult development. In the 18 day period between pupal ecdysis and adult eclosion, vitellogenin was first detected in the blood with this technique 3 days before eclosion. During adult life, as shown in Figure 5, the yolk protein comprised approximately 15% of total blood protein which stained with Coomassie Brilliant Blue. This stain is quite sensitive and will detect less than 0.5 μg of protein in a single band (Weber, *et al.*, 1972).

Figure 6 shows the densitometer tracings from gels of female blood taken just after eclosion from an intact female and from one which had been allatectomized as an early pupa. The vitellogenin band was present in a similar concentration in both. Figure 7 shows the densitometer tracings from gels of homogenates of immature follicles (1 mm long) of intact or of allatectomized females at the time of eclosion. There were no major differences between the two, indicating that vitellogenesis proceeded normally, at least up to this critical stage of development.

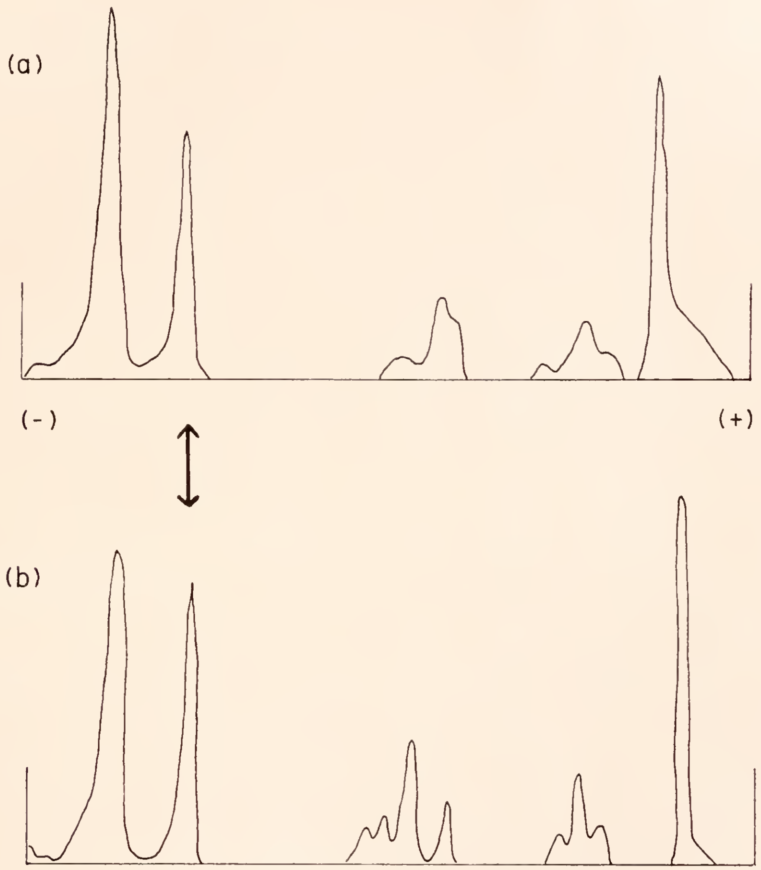

Figure 6. Densitometer tracings of gels of hemolymph from (a) intact female and (b) female allatectomized as pupa. Blood samples were taken immediately after eclosion. Arrow indicates vitellogenin.

Discussion

The results of the experiments reported here confirm that adult *Manduca sexta* females require JH for complete egg maturation. The CA, which cease secretion in the fifth instar larva (Nijhout and Williams, 1974) and remain quiescent during adult development, become active at about the time of eclosion in female moths. This activation of the CA in adults appears to be controlled by the brain as brainless animals do not mature eggs. Yet they will respond to appropriate doses of exogenous JH by making twice the number of eggs found in intact

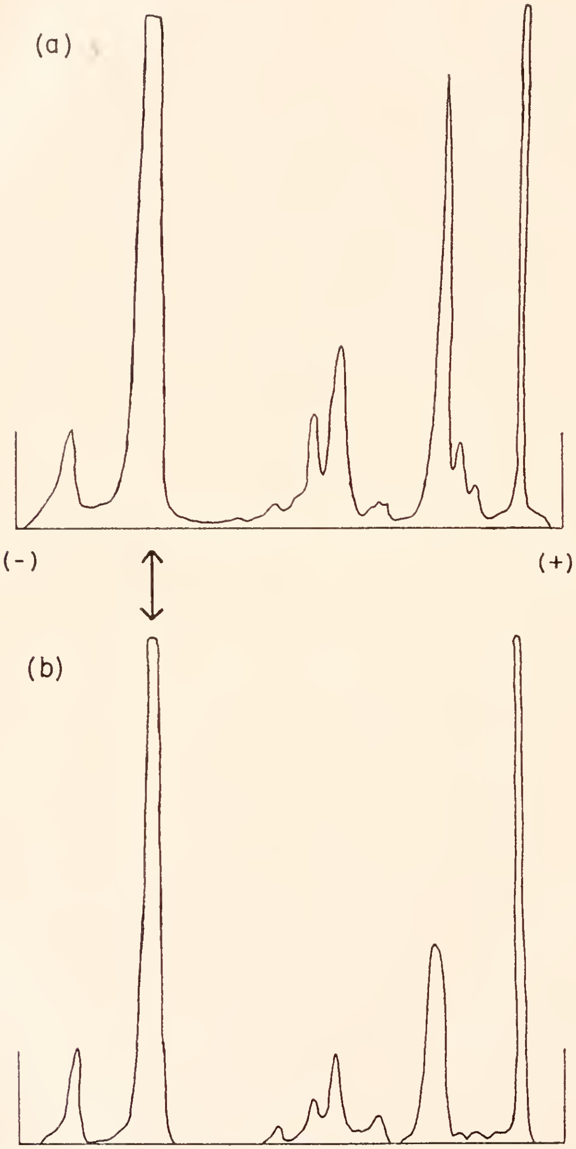

Figure 7. Densitometer tracings of homogenates of immature follicles, 1 mm long from (a) intact female and (b) female allatectomized as pupa. Follicles were dissected at the time of eclosion. Arrow indicates vitellogenin.

females. Therefore, the brain somehow activates the CA but plays no further role in ovarian development.

The egg maturation assay indicates that the various juvenile hormone compounds produced by adult female *Manduca* CA do not have similar activities. Judy *et al.* (1973) found that intact glands, *in vitro*, secreted C-16 and C-17 JH into the medium. Reibstein and Law (1973) have reported that homogenates of adult female glands produced C-18 JH as well. Our results, along with those of Truman *et al.* (1973) and Riddiford and Ajami (1973) would support C-17 and/or C-18 as the active hormone(s) in this species.

Once activated, the CA continue to release JH for about two days in virgin, unfed females. Apparently, about this time, the glands shut off since the rate of egg maturation in an intact female decreases from 3.2 to 0.5 eggs/hour at 48 hours after eclosion (Figure 3C). Similarly, females decapitated before the end of the second day do not form the maximum number of eggs, whereas those decapitated on the third day or later mature the normal complement (Figure 3B). The decreased rate of egg maturation in intact females cannot be due to a depletion of nutritional reserves as Figure 2 shows that unfed females are capable of making up to 300 eggs in 4 days when given an appropriate dose of JH. The JH was injected in a light mineral oil vehicle so as to allow a slow continuous release of hormone into the blood (Riddiford and Ajami, 1973); thus, with the optimum doses, approximately 3.0 eggs/hour were matured during the entire period following injection.

The mechanism which results in the relative inactivity of the CA after the second day of virgin life is unknown. We suspect that the virgin condition results in the inactivation of glands and that some sort of "mating stimulus" would maintain their activity or reactivate them. Mating stimuli, both mechanical and humoral, which stimulate egg production and/or oviposition are known for various insects (see Engelmann, 1970; deWilde and deLoof, 1973; Riddiford and Ashenhurst, 1973). Often these stimuli act via the brain. In view of the fact that the brain activates the CA in *Manduca*, it may also provide a constant stimulation for them. The necessity for the brain in the maintenance of secretion by the CA would explain why glands implanted into decapitated animals sustain the maturation of only half the normal number of eggs. Reimplantation of CA into animals allatectomized as pupae (which therefore have intact brains) restored normal ovarian development as measured by the increase in weight of the gonad (Sroka and Gilbert, 1971).

The role of JH in adult female *Manduca* is novel when compared to its effects in adults of other species that have been studied. The hormone is not necessary for synthesis and accumulation of vitellogenin in the blood nor for its incorporation into the oocyte of less than 1 mm in length. Yet follicles do not complete development in its absence. A critical period can be defined for each follicle. Independent of JH, a follicle can reach that stage characteristic of the terminal follicles of a newly emerged moth. The follicle is 1 mm long, contains a relatively large amount of yolk in the oocyte, but retains a nurse cell cap. Normal development beyond this critical stage requires JH. In its absence, abnormalities appear in both the nurse cells and oocyte. Eventually, all the contents of the oocyte are resorbed and the follicle shrivels. The critical stage is not a resting stage which lies quiescent until exposed to the hormone. As each follicle reaches and passes the critical period, it degenerates.

Similarly in *Aedes aegypti* (Gwadz and Spielman, 1973), the brain activates the CA soon after emergence in the adult female. JH then acts, perhaps directly on the immature follicles, to allow their development to the normal resting stage. From this point, stimuli initiated by the blood meal are needed to begin vitellogenesis. The temporal sequence in *Manduca* female moths is the same except that at the time of eclosion, terminal follicles have already completed most of vitellogenesis. JH acts at this point to allow normal development beyond the critical stage.

Our results are consistent with the hypothesis that JH either acts directly on the developing follicle or that it drives an extra-ovarian process which maintains the correct environment for follicular development. It is obvious that JH is not a trigger for an event or series of events, which, once initiated, no longer require the presence of the hormone to allow the female to mature 100 eggs. If this were true, Curve B in Figure 3 would rise more sharply with respect to Curve C. The fact that the curves are parallel over most of their length indicates that the release of hormone itself is the rate limiting step. We do not know the length of the JH-dependent stage for each follicle; but once the follicle becomes independent of the hormone, it requires a maxium of 12 more hours to reach maturity.

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SUMMARY

1. In *Manduca sexta* females, follicles develop independently of the corpora allata until they reach a critical stage in late vitellogenesis. In the absence of juvenile hormone (JH), follicles then degenerate. When JH is present, the follicles continue development; they then become independent of JH for the final 12 hours of egg maturation.

2. Allatectomy of the pupa of *Manduca sexta* or the newly eclosed adult female prevents egg maturation as does extirpation of the brain from the pupa or decapitation of the adult. The ability to develop follicles beyond the vitellogenic phase is restored by injection of synthetic JH or JH mimics.

3. The brain activates the corpora allata close to the time of adult emergence. The glands secrete JH at a constant rate for 48 hours and then become less active in virgin, unfed moths.

4. A female specific protein, identified by SDS gel electrophoresis, is present in adult blood and egg homogenates. Allatectomy has no effect on the concentration of vitellogenin in the blood nor on its incorporation into immature follicles less than one mm long.

5. The action of JH in promoting egg maturation in *Manduca sexta* appears to be different from that in most other well-studied insect systems in that the hormone is not required for the synthesis and/or uptake of the female-specific yolk protein. Rather it is required for a critical step in development.

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PROTEIN POLYMORPHISMS IN THE HARD CLAMS *MERCENARIA MERCENARIA* AND *MERCENARIA CAMPECHIENSIS*

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The northern hard clam, *Mercenaria mercenaria*, is found intertidally and in shallow waters from the Gulf of St. Lawrence to the Gulf of Mexico. The southern hard clam, *M. campechiensis*, ranges from New Jersey to the Gulf of Mexico and the West Indies (Menzel, 1969). It is found offshore in the northern half of its range and both inshore and offshore in the southern half. Adults of both species are non-mobile and long-lived, surviving up to 20 years. They lie slightly buried in sandy or muddy bottoms and filter the overlying water to obtain food. These bivalves are highly prolific and their larvae are broadcast over wide areas. An average female may produce 20 to 30 million eggs during a summer (Ansell, 1967; Davis and Chanley, 1956). The eggs and sperm are shed into the overlying water where fertilization occurs. Larvae develop in the water column for a period of a week or more (Carriker, 1961). During this time they may be dispersed great distances by water movements. Ample opportunity exists for genetic exchange among adjacent populations.

This paper reports preliminary observations on the population genetics of these important representatives of the estuarine benthic community. Data were obtained by studying the electrophoretically resolvable forms of the hard clam's enzymes. The use of isozymes to answer genetic questions was popularized by Hubby and Lewontin (1966). The rationale for this approach was advanced by Shaw (1965), Gooch and Schopf (1970) and others. Briefly, since the electrophoretic mobility of an enzyme is determined by its primary structure, any enzyme variants detected by electrophoresis are equated with alleles at the locus controlling the primary structure of that enzyme. Hence, with this technique, phenotypic differences reflect genotypic differences at single loci. Such measures of genetic variation may be used to explore a host of previously unanswerable questions.

The questions posed in this study were: (1) how much variation exists within the gene pool of *M. mercenaria* and *M. campechiensis* populations, (2) what genetic differences exist among widely separated populations of *M. mercenaria* and *M. campechiensis*, and (3) does the gene pool of *M. mercenaria* differ greatly from that of its southern congener, *M. campechiensis*.

Genetic data were obtained for the following enzymes: malate dehydrogenase-NAD form (MDH); and two enzymes of unknown substrate, tetrazolium oxidase (TO) and an esterase (EST). Lactate dehydrogenase (LDH) data were treated in a previous publication (Pesch, 1972) but some of that information will be included here for discussion.

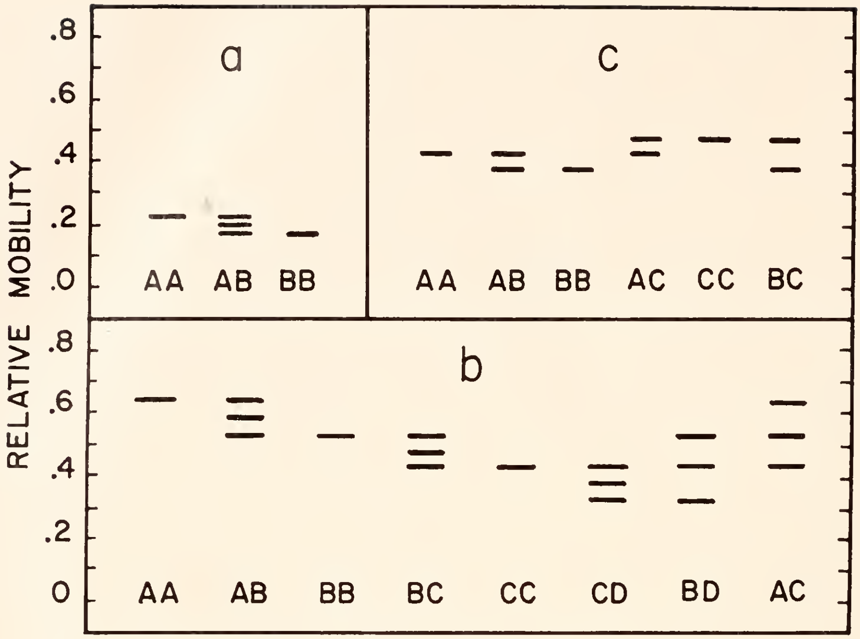

FIGURE 1. Phenotypes observed in populations of *M. mercenaria* and *M. campechiensis;* (a) NAD—Malate dehydrogenase, (b) tetrazolium oxidase and (c) an esterase. Anode is at top. Band mobilities were measured relative to bromphenol blue dye front.

MATERIALS AND METHODS

Four populations of *M. mercenaria* were sampled: one each from the Bideford River, Prince Edward Island, Canada; Boothbay Harbor, Maine; Narragansett Bay, Rhode Island; and Wadmalaw Island, South Carolina. For comparison, two populations of *M. campechiensis,* a southern congener, were sampled: one from Shackleford Banks near Beaufort, North Carolina, and one from Tampa Bay on Florida's Gulf Coast. The samples were collected during the summer of 1970 and included animals from 6.2 to 12.7 cm in length with approximately equal proportions of sexes. A minimum of 50 animals from each population was assayed for each of the enzymes. A pooled sample of Rhode Island *M. mercenaria* provided a reference to compare band mobilities of the enzymes among the populations studied. It is assummed that some forms of the enzymes studied are common to both species because of the identical electrophoretic mobilities of these forms. Enzyme patterns were photographed with Kodak Plus X Pan film.

Preparation of tissue extracts

Tissues were homogenized and extracted in a Tris buffered sucrose solution as described by Pesch (1972). Electrophoretic patterns were stable for several months in extracts stored at −20°. Gill, mantle, muscle and whole animal homogenates were tested. The esterase patterns studied were observed in gill tissue

TABLE I

Malate dehydrogenase NAD form (MDH) alleles and phenotypes from four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis
Phenotypic frequencies observed (expected)

Genotypes	*M. mercenaria*				*M. campechiensis*	
	Can.	Me.	R. I.	S. C.	N. C.	Fla.
AA*	50 (50.0)	47 (46.1)	58 (58.0)	55 (55.0)	58 (58.0)	52 (52.0)
BB		1 (0.1)		0 (0)		
AB		2 (3.8)		1 (0.9)		
	50	50	58	56	58	52
Allelic frequencies						
A	1.00	0.96	1.00	0.99	1.00	1.00
B		0.04		0.01		

* Alleles are arbitrarily labelled with letters.

only; the other enzymes were observed in all tissues tested. Mantle tissue arbitrarily was chosen for the remaining enzyme assays.

Electrophoretic and staining techniques

Disc-polyacrylamide gel electrophoresis was performed on an EC Apparatus vertical cell (model EC 470) as described by Pesch (1972). Buffers for electrophoresis and stains for enzyme demonstration were as follows: *Malate dehydrogenase—NAD form (MDH)*; buffers; same as described by Pesch (1972) for analysis of LDH; stain; nitro blue tetrazolium method modified from Latner and Skillen (1968) by deletion of phenazine methosulfate. *Tetrazolium oxidase (TO)*; buffers; same as for MDH; stain: same as for MDH but with a trace of phenazine methosulfate added. *Esterase (EST)*; buffers; stacking gel buffer same as for MDH, running gel buffer 0.124 M Tris adjusted to pH 7.8 with HCL. Electrode buffer 0.004 M Tris adjusted to pH 8.0 with glycine; Stain; Fast Blue RR salt method with α-naphthyl acetate substrate as described by Latner and Skillen (1968).

RESULTS

Malate dehydrogenase—NAD from (MDH)

Phenotypes for MDH are either one or three bands with no differences between sexes (Fig. 1a). All but four of 324 individuals sampled had a single band of identical electrophoretic mobility (Table I). These were presumably homozygotes for a common allele. One individual had a single band of lesser mobility and was presumably a homozygote for a different allele. Three individuals had both of the above bands plus one of intermediate mobility. These three are thought to be heterozygotes containing both alleles. This enzyme appears to be a dimer with single locus autosomal inheritance.

TABLE II

Tetrazolium oxidase alleles and phenotypes from four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis
Phenotypic frequencies observed (expected)

Genotypes	*M. mercenaria*				*M. campechiensis*	
	Can.	Me.	R. I.	S. C.	N. C.	Fla.
AA	2 (2.4)	18 (15.7)	28 (28.8)	28 (26.6)	1 (1.9)	2 (0.9)
BB	30 (30.4)	12 (9.7)	3 (3.7)	6 (5.0)	14 (12.7)	17 (18.1)
CC			0 (0)	0 (0)	6 (4.8)	2 (3.8)
DD					0 (0)	0 (0)
AB	18 (17.2)	20 (24.6)	22 (20.5)	21 (23.2)	11 (9.9)	6 (8.0)
AC			1 (0.8)	0 (0.8)	7 (6.0)	4 (3.7)
AD					0 (0.4)	0 (0.1)
BC			0 (0.3)	1 (0.3)	12 (15.6)	20 (16.6)
BD					1 (1.0)	1 (0.6)
					1 (0.6)	0 (0.3)
	50	50	54	56	53	52
Allelic frequencies						
A	0.22	0.56	0.73	0.69	0.19	0.13
B	0.78	0.44	0.26	0.30	0.49	0.59
C			0.01	0.01	0.30	0.27
D					0.02	0.01

Tetrazolium oxidase

The metabolic substrate of this enzyme is not known. The oxidase is visualized as light bands on a dark blue tetrazolium background. Patterns consisting of one or three bands are found (Fig. 1b). Single banded patterns are thought to be from homozygotes. Patterns with three bands had two bands with mobilities corresponding to the bands from homozygotes and a third band of intermediate mobility. These are thought to be from heterozygotes. This enzyme is probably a dimer inherited at a single locus with multiple alleles.

A north-south allelic cline paralleling that found for LDH (Pesch, 1972) is suggested for this enzyme (Table II). Two alleles appear in the Canadian population of *M. mercenaria.* The number of alleles increases to four in the Florida population of *M. campechiensis.* In all six populations the phenotypic frequencies fit the Hardy-Weinberg equilibrium model. Only phenotypes with expected frequencies of 10% (5 individuals) or more per population were included in chi-square tests. Tests of significance were made at the $P = 0.05$ level.

Esterase

Esterase enzymes have broad substrate specificities. The assay, with α-naphthyl acetate as substrate, yielded a bewildering array of patterns on electropherograms of gill tissue. However, prominent dark bands of medium mobility consisently were present in patterns explainable by the genetic mechanism of

Table III

Esterase (α-napthyl acetate) alleles and phenotypes from four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis
Phenotypic frequencies observed (expected)

Genotypes	*M. mercenaria*				*M. campechiensis*	
	Can.	Me.	R. I.	S. C.	N. C.	Fla.
AA	42 (41.6)	44 (41.5)	40 (37.9)	54 (54.0)	46 (44.7)	43 (42.0)
BB	0 (0)	2 (0.1)			0 (0.1)	2 (1.0)
CC	1 (0.4)	1 (0.2)	3 (0.9)	0 (0)	1 (0.4)	
AB	2 (1.8)	0 (3.6)			3 (3.5)	11 (13.0)
AC	7 (8.1)	4 (5.4)	7 (11.3)	2 (2.0)	6 (8.0)	
BC	0 (0.2)	0 (0.2)			1 (0.3)	
	52	51	50	56	57	56
Allelic frequencies						
A	0.89	0.90	0.87	0.98	0.88	0.87
B	0.02	0.04			0.04	0.13
C	0.09	0.06	0.13	0.02	0.08	

multiple alleles at a single locus. To facilitate interpretation of highly polymorphic electropherograms, only these dark bands were considered.

Individuals had either one or two dark bands (Fig. 1c). Phenotypes with one band were assumed to be homozygotes, those with two bands heterozygotes. All six populations displayed at least two alleles but a single common allele predominanted (Table II). Phenotypic frequencies fit those predicted by the Hardy-Weinberg equilibrium model for each population.

Discussion

Intrapopulation variance

General conclusions about the genome are made with reservation since only four loci were considered. Obviously more loci need to be examined. However, with at least three of four loci polymorphic in all populations examined (Table IV) it seems safe to infer that the level of genetic variability of these species approaches that of other species.

Lewontin and Hubby (1966) in their classic study of *Drosophila pseudoobscura* estimated that 39% of the loci in the genome were polymorphic for the whole species with an average of 30% of all loci polymorphic for any given population. These estimates were based on data for 21 loci. Selander and Yang (1969), using data from 40 loci, estimated levels of genetic polymorphism in a wild population of the house mouse, *Mus musculus*, and obtained results remarkably similar to those of Lewontin and Hubby (1966). Several marine invertebrates have been examined in similar fashion. Selander *et. al.* (1970) studied 25 loci in 64 individual horseshoe crabs (*Limulus polyphemus*) from four localities on the East and Gulf Coasts. They estimated (page 412) that "single populations of *Limulus*

TABLE IV

Number of alleles found at four loci in four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis

	MDH	EST	TO	LDH	Total	Alleles/Locus
M. mercenaria						
Can.	1	3	2	2	8	2.00
Me.	2	3	2	4	11	2.75
R. I.	1	2	3	5	11	2.75
S. C.	2	2	3	5	12	3.00
M. campechiensis						
N. C.	1	3	4	6	14	3.50
Fla.	1	2	4	7	14	3.50

are, on the average, polymorphic at 25.0% of their loci." Gooch and Schopf (1970) report genetic variability in two species of ectroprocts found in the vicinity of Woods Hole, Massachusetts. *Schizoporella unicornis* had two of eight loci polymorphic (25%) while *Bugula stolonifera* had 6 of 11 loci polymorphic (54.5%).

Both populations of *M. campechiensis* have a total of 14 alleles segregating at four loci for an average of 3.5 alleles per locus. The Canadian, Maine, Rhode Island, and South Carolina populations of M. mercenaria averaged 2.0, 2.75, 2.75, and 3.0 alleles per locus, respectively. These compare with 2.0 alleles per locus for *D. pseudoobscura* (Hubby and Lewontin, 1966) and 2.25 alleles per locus for *M. musculus* (Selander and Yang, 1969).

If the variation contained in the genome of *Mercenaria* does approach the level found in other species, a wide variety of genotypic combinations is potentially possible. The reproductive rate of the hard clam is geared to exploit this potential. An average adult female produces approximately 25 million eggs during a single sqawning season (Davis and Chanley, 1956). High fecundity permits re-shuffling of genetic material within a population. Dispersal, via planktonic larvae, permits maintenance of variation by genetic exchange among populations.

Interpopulation variance

The sampling transect extended from Prince Edward Island, Canada to Tampa Bay, Florida. Over this range, three patterns were notable in the data (Table IV). Monomorphism is approached by the MDH locus with a single allele perdominating in all populations. Polymorphism is pronounced at the EST locus but the data show no patterned shift. Clines were observed at both the TO and the LDH loci.

Gooch, Smith and Knupp (1972) conducted a similar survey on the estuarine mud snail *Nassarius obsoletus*. Their sampling transect extended from Cape Cod, Massachusetts to Beaufort, North Carolina, which overlaps the middle third of the present survey. Six loci were examined in the mud snail; four were monomorphic; two were polymorphic. Gene frequencies were uniform along the transect. Interestingly, three enzymes (MDH, TO, and LDH) were examined in both surveys. In the mud snail MDH was monomorphic, but with an aberrant band present in 1 to 2 per cent of the individuals. The patterns for the hard clam MDH are similar, however the low frequency aberrant bands are identifiable as second alleles. Tetrazolium oxidase of the mud snail was present consistently in two forms. The authors regarded these as representing separate loci monomorphic for single alleles. In the hard clam TO is controlled at a single locus and is clearly polymorphic, displaying a latitudinal cline. Lactate dehydrogenase was found to be polymorphic in both studies. In the mud snail three alleles were found in uniform frequencies along the transect. In the hard clam LDH displayed a marked latitudinal cline.

Both the mud snail and the hard clam are physiologically tough, surviving in a diversity of environments over broad geographic ranges. The mud snail presents a uniform genetic picture while the hard clam presents a varied array of genetic patterns. It seems they have followed separate genetic routes to achieve durability. Lewontin (1957) suggests two routes a population may take to survive a change in environment. The first is individual homeostasis. A population survives because each of its members is able to survive in a variety of environments. Heterozygosis is thought to be the genetic basis of such homeostasis. Populational homeostasis is the second route to survival. The genetic composition of a population is diverse, containing an array of highly varied genotypes. Each genotype would have a distinct range of environmental limits so that the sum of a variety of genotypes would have a greater range of survival than any single genotype. At least some of these genotypes would be able to survive even drastic environmental changes.

The mud snail presents a uniform genetic picture, suggesting that this species survives by the route of individual homeostasis. The individual is "optimized." The hard clam presents a varied genetic picture suggesting that they survive by the route of populational homeostasis. A diverse genetic composition exploited by high fecundity produces an almost infinite array of genotypes.

Gooch *et. al.* (1972) ascribe the genetic uniformity of the mud snail to either mechanisms of balancing selection, pervasive gene flow or a combination of these. They find it difficult to accept the mechanism of balancing selection because of the varied environments represented by their samples. Instead they argue in favor of extensive gene flow, citing the vector of planktonic larvae. However, this vector is common also to the hard clam.

A study of direct relevance was reported by Levinton (1973). He tested the role of environmental variability in regulating genetic variability. Six bivalve mollusk species were selected from (page 75) "an inferred gradient of environmental variability." Estimates of genetic variability were based on polymorphisms observed in two enzymes, phosphohexose isomerase (PHI) and leucine aminopeptidase (LAP). He concluded (page 76) "it seems clear that species supposed to be experiencing more environmental variability are more polymorphic than those living in more constant environments." Two explanations were considered. One is selection for heterozygotes in a varying environment. The second is diversifying selection in a heterogeneous environment with extensive gene flow.

Levinton (1973) champions the view that spatial and temporal environmental variances selectively enhance the amount of genetic variation in a population. A second view holds that selectively neutral alleles are important (Kimura, 1968; King and Jukes, 1969). Pertinent to this view is a study reported by Schopf & Gooch (1971). A sampling of deep-sea invertebrates, representing a very stable environment, revealed a high level of genetic variability. These data are most easily explained by assuming the presence of selectively neutral alleles.

TABLE V

Percentage of heterozygotic genotypes found at four loci in four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis

	MDH	EST	TO	LDH	Mean
M. mercenaria					
Can.	0	17.3	36.0	100	38.33
Me.	4.0	7.8	40.0	86.0	34.45
R. I.	0	14.0	42.6	83.6	35.05
S. C.	1.8	3.6	41.1	82.7	32.30
M. campechiensis					
N. C.	0	17.5	60.4	41.5	29.85
Fla.	0	19.6	59.6	70.6	37.45
Mean	0.97	13.30	46.62	77.40	34.57

The hard clam data are not so easily explained. The presence of clines at two of four loci (TO, LDH) suggests adaptive significance. The high number of alleles in the southern populations may arise from gene flow within a spatially heterogeneous environment or from the presence of neutral alleles surviving in a relatively more benign, stable environment. Either source is a possibility. Contrasting data exist on the occurrence of heterozygotes (Table V). The loci for MDH and EST show no trends. Tetrazolium oxidase is characterized by an increasing number of heterozygotes in the southern populations. This is an expected consequence of an increased number of alleles. Lactate dehydrogenase has a maximum of heterozygotes in the Canadian population. This trend may reflect increased selection for heterozygotes at this locus by fluctuating environments. The total picture presented by the hard clam data suggests a complex of selective processes probably of differing biological significance.

Species comparison

Mercenaria mercenaria, the northern hard clam, and *Mercenaria campechiensis*, the southern hard clam, along with several subspecies, occur along the Atlantic and Gulf Coasts of North America (Menzel, 1969). *M. mercenaria* is confined to inshore waters from Canada to the Gulf of Mexico. *M. campechiensis* occurs offshore in the northern part of its range, inshore south of Cape Kennedy, and in both habitats in the Gulf of Mexico.

Menzel (1969) concluded that the subspecies *M. mercenaria notata* and *M. mercenaria alba* had no validity, but suggests that the subspecies *M. mercenaria texana* is a naturally occurring hybrid between the two species. *M. mercenaria* and *M. campechiensis* hybridize readily in the lab and have been reared through the F_2 generation. "Shell morphology of about $\frac{3}{4}$ of the F_2's is very similar to the subspecies *M. mercenaria texana* . . ." (Menzel, 1969, page 8).

The ease of raising hybrids in the lab and the presence of intermediates in the field suggest that gene flow may occur between these species. Menzel and Menzel (1965) studied the chromosome complements of both species and their hybrids. They conclude (page 187) that the "homology and regular behavior of the

TABLE VI

Allelic overlap (%) at four loci among four populations of Mercenaria mercenaria *and two populations of* Mercenaria campechiensis

	M. campechiensis		*M. mercenaria*		
	Fla.	N. C.	S. C.	R. I.	Me.
M. mercenaria					
Can.	47	57	54	58	73
Me.	56	67	77	69	
R. I.	67	79	92		
S. C.	63	73			
M. campechiensis					
N. C.	87				

chromosomes of the two species, revealed at meiosis in F_1 hybrid, demonstrate that there is no gross chromosomal barrier to such gene interchange."

In the present study the gene pools of these species are seen to have many alleles in common (Table VI). Of a total 16 alleles observed at four loci in four populations of *M. mercenaria* and two populations of *M. campechiensis,* 12 alleles (75%) are found in both species. Indeed the genetic differences among populations of *M. mercenaria* are greater than differences between the species. For example, comparison of the Canadian and South Carolina populations of *M. mercenaria* revealed that from a total of 13 alleles they shared 7 (54%) in common. The accumulated evidence suggests that *M. mercenaria* and *M. campechiensis* probably represent what Dobzhansky (1970) termed "incipient species." These two species, recently derived from an ancestral line, have not yet reached reproductive isolation.

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SUMMARY

Four gene loci were characterized by polyacrylamide disc-gel electrophoresis of four enzymes in the hard clams, *M. mercenaria* and *M. campechiensis.* Four populations of *M. mercenaria* and two populations of *M. campechiensis* were se-

lected from a transect between Prince Edward Island, Canada and Tampa Bay, Florida. Three of the four enzymes were polymorphic in all six populations. *M. mercenaria* averaged 2.6 alleles per locus for four populations while *M. campechiensis* averaged 3.5 alleles per locus for two populations. Malate dehydrogenase—NAD form (MDH) had a single allele predominating in all populations. A second rare allele appeared in the Maine and South Carolina populations of *M. mercenaria*. Polymorphism was pronounced at an esterase (EST) locus but the data showed no patterned shift along the transect. North to south clines were observed at both the tetrazolium oxidase (TO) and the lactate dehydrogenase (LDH) loci. The different patterns observed for each locus suggest a complex of selective processes probably of differing adaptive significance. In the portion of the gene pools studied these species were found to have many alleles in common (12 of 16). Commonality of alleles, homology of chromosomes, ease of hybridization, and intergrades in the field suggest that these species have not yet reached reproductive isolation.

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STUDIES ON THE BIOLOGICAL PROPERTIES OF COELOMIC FLUID OF SEA URCHIN. II. NATURALLY OCCURRING HEMAGGLUTININ IN SEA URCHIN

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It is well known that invertebrates may have hemagglutinins for a variety of erythrocytes (Huff, 1940; Tyler, 1946; Cushing, Calaprice and Trump, 1963; Brown, Almodovar, Bhatia and Boyd, 1968; Prokop, Uhlenbruck and Köhler, 1968; McKay, Jenkin and Rowley, 1969).

Recently, in the course of studies on the biological properties of coelomic fluid from sea urchins (*Anthocidaris crassipina, Pseudocentrotus depressus,* and *Hemicentrotus pulcherrimus*), it was demonstrated that the fluid was highly effective to lyse erythrocytes from some species of animals and that the nature of its active principle may be a protein or a protein-like substance (Ryoyama, 1973). Soon afterward, it was found that the same materials from these sea urchins also exhibited hemagglutinating activity against rabbit erythrocytes. Although natural hemagglutinin can be found in the coelomic fluid of sea urchin, its chemical and biological properties have been poorly understood (Tyler, 1946; Brown, *et al.*, 1968; Uhlenbruck, Sprenger and Heggen, 1970). The present report gives data on the occurrence of hemagglutinins in the coelomic fluid of sea urchins stated above, and on some properties of their hemagglutinins.

MATERIALS AND METHODS

Three species of sea urchin, *Anthocidaris crassispina* (Agassiz), *Pseudocentrotus depressus* (Agassiz), and *Hemicentrotus pulcherrimus* (Agassiz) were used. They were harvested at three different littoral areas (Ogi and Kabuto in Ishikawa Prefecture, and Amabarashi in Toyama Prefecture, Japan). The coelomic fluid preparation was obtained by the method reported previously (Ryoyama, 1973). The fluid (protein content, 467 μg/ml) pretreated at 56° C for 30 minutes to suppress their hemolytic activity was used throughout the experiments, except for heat stability test of the hemagglutinating activity. The protein content of the coelomic fluid preparation was determined by the Folin-phenol method (Lowry, Rosebrough, Farr, and Randall, 1951), using bovine albumin as a reference standard.

The erythrocytes from various animals were centrifuged at $250 \times g$ and suspended in 0.05 M Tris-HCl buffer solution (pH 8.0) containing 0.15 M NaCl and 0.02 M $CaCl_2$ (Tris-NaCl-$CaCl_2$ buffer solution). Unless otherwise stated, this buffer solution was used for dialyzing coelomic fluid, diluting the dialyzed fluid preparation, and also for suspending erythrocytes. The final concentration of erythrocytes was adjusted to $1.0–1.6 \times 10^8$ cells per ml.

To serial two-fold dilutions of the coelomic fluid preparation, an equal volume

TABLE I

Relative hemagglutinating activity of coelomic fluid preparation obtained from three different species of sea urchin against erythrocytes from various species of animals

Erythrocyte species	Hemagglutinating titer*		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
Human-Blood group A	2^5	2^3	2^3
B	2^4	2^4	2^4
O	2^3	2^2	2^3
Rabbit	2^9	2^6	2^9
Mouse	<2	<2	<2
Guinea pig	<2	2^3	2^5
Dog	2^6	<2	2^6
Sheep	<2	<2	<2

* Represented as the reciprocal of the highest dilution effecting agglutination.

of erythrocyte suspension was added. After mixing, each of the test tubes was incubated at 37° C for 30 minutes and hemagglutination was examined macroscopically or microscopically.

Trypsin (Difco, 1 : 250) and Bromelains (from pineapple) from the Nutritional Biochemical Corp. were used.

RESULTS

Effect of coelomic fluid preparation against erthyroctes from different species of animals

The results obtained in the experiments to examine the susceptibility of erythrocytes from different sources to coelomic fluid preparation from sea urchin are presented in Table I. Rabbit erythrocytes were found to agglutinate when they were mixed with the coelomic fluid preparation. Hemagglutinin titers of *A. crassispina* and *H. pulcherrimus* against rabbit cells were 2^8 to 2^9 and that of *P. depressus* was 2^4 to 2^6. The coelomic fluid from *H. pulcherrimus* reacted more actively than those from the other two species against rabbit cells, though the titers of agglutination by *H. pulcherrimus* and *A. crassispina* were almost the same. Human erythrocytes were also sensitive to these coelomic fluid preparations, and their hemagglutinating activities were much the same. However, some differences in susceptibility against the fluids were found among the type of blood group (A, B, O) in man; A- and B-type cells were more sensitive than O-type cells. The buffer solution itself used in these experiments had no effect on erythrocytes. There were some variations in agglutinating activity among species of sea urchin. The coelomic fluid preparation from *A. crassispina* agglutinated dog erythrocytes but that from *P. depressus* did not. Conversely, the fluid from *P. depressus* agglutinated guinea pig erythrocytes but that from *A. crassispina* did not. The fluid from *H. pulcherrimus* equally produced hemagglutination of erythrocytes from both animals. On the other hand, three coelomic fluid preparations did not hemagglutinate either sheep or mouse erythrocytes.

TABLE II

Effect of bivalent cations on the hemagglutinating activity of 3 coelomic fluid preparations against rabbit erythrocytes

Final concentration of metallic salt	Hemagglutinating titer		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
None	$<2^2$	$<2^2$	$<2^2$
10 mM $CaCl_2$	2^7	2^6	2^7
20 mM $CaCl_2$	2^8	2^6	2^8
100 mM $CaCl_2$	2^8	2^6	2^8
20 mM $MgCl_2$	$<2^2$	$<2^2$	$<2^2$
20 mM $MgCl_2$ + 20 mM $CaCl_2$	2^7	2^6	2^7
20 mM $CaCl_2$ + 20 mM EDTA	$<2^2$	$<2^2$	$<2^2$
40 mM $CaCl_2$ + 20 mM EDTA	2^7	2^6	2^6

In addition, hemagglutinating activity of coelomic fluid was not dependent on age (size of shell) or sex of sea urchin, but might be dependent on the season of collection; in the case of *A. crassispina*, all the specimens collected during April and May showed hemagglutinating activity, a few of the specimens collected during July and August did not show this activity, and only a few of the specimens collected in November showed this activity. (The spawning season of this species is June to August). Moreover, hemagglutinating spectra of coelomic fluids from specimens collected in different places were also similar to that presented in Table I.

The sea water of the place where sea urchin was collected was almost entirely ineffective to erythrocytes, even when concentrated sea water was used.

Furthermore, no difference in the hemagglutinating potency of the coelomic fluid preparation was observed in the range of 1° C to 37° C and pH 6.6 to 9.3. Therefore, it may be said that exhibition of hemagglutinination by the fluid preparation is not dependent on temperature. On the other hand, the agglutinating activity of the fluid from *P. depressus* was found to be slightly higher at 4° C than at 37° C against guinea pig erythrocytes.

Since rabbit cells among the tested erythrocytes were found to be the most susceptible to coelomic fluid preparation from sea urchin, the following experiments were carried out mainly on rabbit cells.

Effect of calcium on the hemagglutinating activity of coelomic fluid preparation

A preliminary experiment had demonstrated that calcium ion brought a marked increase of hemagglutinating titer of coelomic fluid preparation. Further investigations on the enhancing effect of calcium ion was repeated employing the following system: A mixture of 0.1 ml of coelomic fluid preparation (dialyzed against 0.85% NaCl solution), which was diluted two-fold serially with 0.85% NaCl solution, 0.1 ml of rabbit erythrocytes suspended in Tris-NaCl buffer solution, and 0.2 ml metallic salt in the same buffer solution was incubated at 37° C for 30 minutes.

As shown in Table II, addition of $CaCl_2$ to this hemagglutination test system greatly increased the activity of coelomic fluid preparation. No agglutination was observed in the absence of coelomic fluid preparation or in the presence of $CaCl_2$ alone. The addition of EDTA abolished this effect, which was regained on further addition of calcium. Magnesium had no effect on hemagglutination and also did not interfere with the enhacing effect of calcium. Enhancement of hemagglutinating activity of coelomic fluid preparation by calcium ion was also observed in the experiment with guinea pig and human erythrocytes.

It thus appears that calcium is essential for hemagglutination produced by coelomic fluid preparation from three different species of sea urchin.

Effect of temperature and pH on the stability of coelomic fluid preparation

In order to examine the effect of temperature on the stability of hemagglutinating activity of coelomic fluid preparation, aliquots of the fluid were placed in a water bath in the range of 1 to 100° C for 30 minutes, and hemagglutinating activity was then estimated. In the case of *A. crassispina*, hemagglutinating activity of the coelomc fluid preparation was not affected in the temperature range of 1 to 65° C, while its activity was almost completely destroyed at 70° C. In the case of *P. depressus,* the activity was not affected to 70° C, but was destroyed (about 50%) at 75 or 80° C, and completely abolished at 85° C. On the other hand, the coelomic fluid preparation from *H. pulcherrimus* was not affected even at 100° C.

When the coelomic fluid preparations dialyzed against 0.85% NaCl solution were placed at 4° C for 12 hours in the buffer solution of various pH values (3.2 to 10.6), no change of the activity was observed at the the pH range stated above. The coelomic fluid preparations from three different species of sea urchin seem to be very stable in the region of these pH values.

Effect of some agents on the stability of coelomic fluid preparation

The coelomic fluid preparations from the three species were digested by proteinase (trypsin, bromelain) in order to ascertain whether or not the factor carring the hemagglutinating activity of each fluid was a protein. The reaction mixture of 0.3 ml of coelomic fluid preparation and 0.3 ml of trypsin solution (1–5 mg/ml) or bromelain solution (0.1–1 mg/ml) was incubated at 37° for 1 hour. To each of the reaction mixtures diluted serially was added an equal volume of erythrocyte suspension, and the mixture was placed at 4° C for 30 minutes to minimize the enzyme action. As the results, trypsin had no effect on hemagglutinating activity. No hemagglutination was observed in the absence of coelomic fluid preparation or in the presence of trypsin alone. Accordingly, hemagglutinating activity of the fluids from three species of sea urchin seems to be resistant to tryptic digestion, while bromelain at 0.1 or 0.05% concentrations brought about a considerable decrease in the activity when the coelomic fluid preparations from *A. crassispina* and *P. depressus* were used. On the other hand, the fluid from *H. pulcherrimus* was not affected by bromelain under the same condition. No hemagglutinination was observed in the presence of bromelain alone. In addition, susceptibility of the rabbit erythrocytes pretreated with bromelain or typsin to coelomic fluid preparation was not impaired but slightly increased. Thus, it was thought that the decrease in the activity was actually due to digestion of the coelomic fluid (from *A. crassispina* and *P. depressus*) by bromelain.

TABLE III

Effect of 2-mercaptoethanol on the hemagglutinating activity of 3 coelomic fluid preparations against rabbit erythrocytes

Concentration (M)	Hemagglutinating titer		
	A. crassispina	*P. depressus*	*H. pulcherrimus*
—	3×2^7	3×2^3	3×2^5
0.2	3×2^7	3×2^2	3×2^4
0.4	$<3 \times 2$	$<3 \times 2$	3×2^2
0.4*	3×2^7	$<3 \times 2$	3×2^4

* Without treatment with sodium iodoacetate.

Further experiment was made to investigate whether or not 2-mercaptoethanol, which is a reagent that breaks up the S-S linkage in protein molecules, affects hemagglutinating activity of coelomic fluid preparation. The experiment was carried out as follows: A mixture of 0.3 ml of 2-mercaptoethanol (0.4–0.2 M) and 0.3 ml of coelomic fluid preparation was incubated at 17° C for 1 hour. To each of the reaction mixtures was added 0.3 ml of sodium iodoacetate solution (0.6–0.3 M), and then placed at 4° C for 12 hours, followed by dialysis against Tris-NaCl-$CaCl_2$ buffer solution. These results are shown in Table III. Hemagglutinating activity of coelomic fluid preparations (from *A. crassispina* and *P. depressus*) was completely destroyed by 0.4 M of 2-mercaptoethanol, but the fluid from *H. pulcherrimus* was slightly affected at the same concentration of the reagent. When the fluids pretreated with 2-mercaptoethanol were dialyzed against Tris-NaCl-$CaCl_2$ buffer solution without iodoacetate treatment, hemagglutinating activity of the fluid from *A. crassispina* was completely restored, but not that of the fluid from *P. depressus*. The treatment with sodium iodoacetate alone did not affect the activity. The effect of periodate on the fluid preparation was examined as follows: A mixture of 0.3 ml of coelomic fluid preparation dialyzed against 0.85% NaCl solution, 0.2 ml of 0.2 M citrate buffer (pH 5.5), and 0.1 ml of various concentration of periodate solution adjusted to pH 5.5 with 2 N NaOH was incubated at 20° C for 4 hours. The reaction mixture was dialyzed overnight against 0.85% NaCl solution and then against Tris-NaCl-$CaCl_2$ buffer solution to remove the periodate, and the activity was estimated. Periodate is known to destroy sugars by oxidation. Hemagglutinating activity of the fluid preparation from *H. pulcherrimus* was not affected even with 100 mM of periodate, whereas that of *A. crassispina* and *P. depressus* was completely destroyed at 25 mM of the reagent. The activity of coelomic fluid treated at these conditions without periodate was not changed.

Inhibition tests with simple sugars

In an attempt to find the chemical nature of specific receptor of sea urchin agglutinins, inhibition tests were carried out with 36 simple sugars. These particular sugars have been generally used to examine the specificity of agglutinins. A number of authors have reported that the specificity of most receptor sites against various agglutinins depends to some degree on the sugar moiety in receptor molecules. From each of these sugars, 0.2 M stock solution in Tris-NaCl-CaCl$_2$ buffer solution was prepared, with the exception of dulcitol, salicin, and raffinose in 0.067 M, and cellobiose, lactose, and D-melezitose in 0.1 M. Equal volumes of the coelomic fluid preparation were added to each serial dilution of the stock sugar solution, and incubated at 20° C for 2 hours. After addition of rabbit erythrocyte suspension, the incubation mixture was reincubated at 37° C for 30 minutes. Test materials of mono-, oligo-, and derived saccharides included D-ribose, 2-deoxy-D-ribose, D-arabinose, L-arabinose, D-xylose, L-xylose, D-glucose, L-glucose, 2-deoxy-D-glucose, D-mannose, D-galactose, D-sorbitol, D-mannitol, dulcitol, inositol, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetyl-D-galactosamine, D-glucosamine HCl, D-mannosamine HCl, D-galactosamine HCl, salicin, L-rhamnose, D-digitoxose, D-fucose, L-fucose, D-fructose, L-sorbose, cellobiose, lactose, maltose, sucrose, trehalose, melibiose, D-melezitose, and raffinose.

Hemagglutination by the coelomic fluid preparations from *A. crassispina* and *H. pulcherrimus* was not inhibited by the tested sugars. No hemagglutination was observed in the presence of the sugar alone. On the other hand, hemagglutination by *P. depressus* was affected by nine sugars; D-galactose, L-glucose, D-digitose, inositol, salicin, lactose, melibiose, D-melezitose and raffinose. Complete inhibition was observed in the concentration range of 0.0125–0.05 M. These results suggested that there might be some correlation between the chemical nature and inhibiting activity in these 9 effective sugars. Inhibition tests with these sugars were made repeatedly and the results were reproducible.

Inhibition test with human saliva

It has already been reported that certain hemagglutinins from marine sources react with human erythrocytes, and hemagglutination by them can be inhibited by human secretor saliva (Brain and Grace, 1968; Pemberton, 1971). In connection with this fact, the effect of human saliva on hemagglutination by the coelomic fluid preparation of sea urchin was examined. The test method was as follows: An equal volume of saliva and the coelomic fluid preparation was incubated at 20° C for 2 hours, rabbit erythrocyte suspension was added, and re-incubated at 20° C for 30 minutes. All of 7 kinds of saliva used (2 of blood group A, 2 of blood group B, and 3 of blood group O) completely inhibited hemagglutination by the fluid of *A. crassispina*, but not those of *P. depressus* and *H. pulcherrimus*. When human erythrocytes instead of rabbit cells were used, hemagglutination by the fluids from three different species of sea urchin was completely inhibited by the saliva materials so far examined. Moreover, inhibitory effect of saliva was observed in agglutination of dog erythrocytes by *A. crassispina* but not by *H. pulcherrimus*, while the saliva slightly inhibited agglutination of guinea pig erythrocytes by *P. depressus* but not by *H. pulcherrimus*. In all these cases, inhibition of hemagglutination by saliva seems not to be related to the difference in blood group of man, and saliva itself had no effect on erythrocytes.

Absorption test

The specificity of hemagglutinin of sea urchin was examined by the absorption method. Tests were scheduled to determine whether the hemagglutinin for rabbit

TABLE IV

Specificity of hemagglutinin

Sea urchin	Erythrocyte used for absorption	Titers of hemagglutinin to			
		Man	Rabbit	Dog	Guinea pig
A. crassispina	No absorption	2^4	2^8	2^5	<2
	Man	<2	<2	<2	—
	Rabbit	<2	<2	<2	—
	Guinea pig	2^2	2^8	2^4	—
	Sheep	2^3	2^8	2^4	—
P. depressus	No absorption	2^5	2^8	<2	2^5
	Man	<2	2^6	—	<2
	Rabbit	<2	<2	—	<2
	Guinea pig	<2	2^6	—	<2
	Sheep	2^3	2^6	—	2^3
H. pulcherrimus	No absorption	2^8	2^{11}	2^9	2^7
	Man	<2	2^9	2^9	2^6
	Rabbit	2^6	<2	<2	<2
	Guinea pig	2^6	2^9	2^8	<2
	Sheep	2^6	2^9	2^8	2^5

erythrocytes was identical to those for others, human, dog or guinea pig erythrocytes, and whether the hemagglutinin for each erythrocytes of the animals was identical to that for rabbit cells. The coelomic fluid preparation from sea urchin was mixed with an equal volume of packed erythrocytes. The mixture, after placing at 20° C for 2 hours, was centrifuged and then the supernatant was used for the hemagglutionation test.

These results are shown in Table IV. It is obvious that the hemagglutinin for rabbit erythrocytes was eliminated through the absorption by human erythrocytes in *A. crassispina*, and *vice versa*. Either rabbit cells or human cells absorbed the hemagglutinin for dog erythrocytes. Therefore, the hemagglutinin of *A. crassispina* is thought to be homogeneous. On the other hand, two hemagglutinins may occur in the coelomic fluid preparation from *P. depressus*. One is only to rabbit erythrocytes and another is common to rabbit, human and guinea pig erythrocytes. It may be assumed that each of these two hemagglutinins reacts to a different site on the surface of rabbit erythrocytes. In the case of *H. pulcherrimus*, three hemagglutinins may occur in this fluid preparation; the hemagglutinin only for human erythrocytes, the hemagglutinin being common to rabbit, dog and guinea pig erythrocytes, and the hemagglutinin being common to rabbit and dog erythrocytes.

In addition, no difference in absorption tests was observed among the types of blood group (A, B, O) in man, and the erythrocytes which could not be agglutinated by the coelomic fluid preparation from sea urchin did not absorb the hemagglutinin of sea urchin.

DISCUSSION

Present results showed that three species of sea urchin possessed hemagglutinins for several mammalian erythrocytes in their coelomic fluid. Rabbit erythrocytes showed the highest susceptibility to three coelomic fluid preparations so far examined. In this case, calcium ion was essential for development of high hemagglutinating potency and the addition of EDTA abolished this activity. Magnesium ion was entirely ineffective. As one of the procedures for the examination of hemagglutinin activity in invertebrates, the use of calcium has been proposed in order to detect the activity (Uhlenbruck, Reifenberg and Heggen, 1970). Marchalonis and Edelman (1968) reported that a hemagglutinin from the hemolymph of the horseshoe crab, *Limulus polyphemus*, is a protein having a molecular weight of approximately 40×10^4, which consists of a number of subunits linked through non-covalent interactions, and that its agglutinating activity is potentiated by calcium but not by magnesium. They suggested that bound calcium may stabilize the native structure of the protein. As described in this paper, some difference was observed in the response of hemagglutinins from the three species of sea urchin to chemical and physical agents. The hemagglutinins from *A. crassispina* and *P. depressus* might be a protein-like substance, because they were fairly unstable to heat treatment, and to bromelain and 2-mercaptoethanol. However, it is unknown that the difference in the effects of bromelain and trypsin is due to either the susceptibility of active sites of hemagglutinin to both enzymes or the necessity of certain molecular size of agglutinin to induce hemagglutination. Their molecular weight was also determined as over 20×10^4 by means of gel filtration on Sephadex G-200 (unpublished data). Considering these facts it seems likely that calcium ion participates in stabilization of native structure of the hemagglutinins, as in the hemagglutinin of horseshoe crab mentioned above. Meanwhile, from the fact that hemagglutinating activity of *H. pulcherrimus* was heat-stable and resistant to proteinase and 2-mercaptoethanol, the active principle was considered to be complicated carbohydrates. Fuke and Sugai (1972) have recently reported that the hemagglutinin of ascidian may be a polysaccharide or a mucopolysaccharide from the fact that it is very stable to heat, is resistant to trypsin digestion, and is destroyed by periodate. The hemagglutinin from *H. pulcherrimus* was very similar in properties to that of ascidian except for resistance against periodate treatment.

Watkins and Morgan (1952) showed first that simple sugars are capable of neutralizing hemagglutinin of an eel. Later, Mäkelä (1957) reported the inhibition of hemagglutination by plant agglutinins (Leguminosae seeds) with simple sugars, and classified the sugars into four groups according to the correlation between plant agglutinin and simple sugars. The hemagglutinin of sea urchin (*A. crassispina* and *H. pulcherrimus*) against rabbit erythrocytes was not inhibited with the simple sugars tested while the hemagglutination by the fluid from *P. depressus* was inhibited by several simple sugars. The inhibition in the case of *P. depressus* was never dependent on Mäkelä's classification. In addition, it is known that simple sugars inhibit only a few animal agglutinins and that plant agglutinins in general neutralize at lower concentrations of simple sugars than animal agglutinins (Mäkelä, 1957; Pemberton, 1969). Since the inhibitory effect of L-fucose was considerably less than the activity of the intact H-substance, Watkins and Morgan (1952) stated that simple sugar alone does not account for the complete specificity of the blood group hapten. Therefore, it might be thought that the receptor site for the hemagglutinin of sea urchin was more complex than that for simple sugars.

The hemagglutinin in coelomic fluid preparation from sea urchin could be absorbed by erythrocytes. This property is similar to that of isohemagglutinin present in mammalian serum. The data of absorption test showed that the hemagglutinin of *A. crassispina* was homogeneous to the erythrocytes tested, and that in the coelomic fluid preparation from *P. depressus* two hemagglutinins occurred. Furthermore, three hemagglutinins could be, at least, found in the coelomic fluid preparation from *H. pulcherrimus*. On the other hand, there was a parallelism between the results of absorption test and those of saliva inhibition test so far examined.

Brown, *et al.* (1968) reported the hemagglutinating activity of coelomic fluids from seven species of sea urchin against human erythrocytes. All of the fluids tested, except that from *Diadema antillarum*, reacted with human cells (A, B, O group) while the fluid from *Strongylocentrotus droebachiensis* did not react with A_1 and O_h (Bombay) cells. In the case of fluids from *Echinarachnius parma* and *S. droebachiensis*, a difference in hemagglutinating activity was found among individuals of specimens; three of 6 in *E. parma* and 5 of 15 in *S. droebachiensis* showed the activity. This is in agreement with the present data that there was a difference in the activity of specimens from *A. crassispina*. The difference in individuals was also reported on hemagglutination by the sera of horseshoe crab (Cohen, Rose and Wissler, 1955). In a previous paper (Ryoyama, 1973) it was shown that the coelomic fluid of sea urchin possessed hemolysin, which was not identical with hemagglutinin, and that the difference of hemolytic activity in individuals was seen, as in hemagglutinating activity reported in this paper. There was no correlation between the occurrence of hemolysin and hemagglutinin in each of the specimens. Brown, *et al.* (1968) also detected hemolytic activity in the fluid from *Lytechinus variegatus* but not in other species which belong to the same order as species we tested.

Tyler (1946) could not detect the hemagglutinating activity in the body fluid of *Strongylocentrotus purpuratus* against guinea pig erythrocytes. It may be thought that this is due to the difference of individuals in the species, or to deficiency of calcium ion in the reaction mixture. Calcium ion was essential for agglutination of guinea pig cells as well as other erythrocytes tested with *H. pulcherrimus*, as presented in this paper, and *S. purpuratus* belongs to the same family as *H. pulcherrimus* in the sea urchin. Therefore, calcium ion seems to be necessary for the hemagglutinating activity of the fluid from *S. purpuratus*.

On the other hand, Uhlenbruck, Sprenger and Heggen (1970) reported that the coelomic fluid from *Psammechinus miliaris* agglutinates human and animal erythrocytes, and that the preparation extracted from this fluid with phenol-KCl reacts with neuraminidase-treated human erythrocytes and pronase-treated cells of bovine and pigeon. Although sheep erythrocytes were not susceptible to the fluids of sea urchins tested, they became susceptible to the fluid of *A. crassispina* when the cells were treated with bromelain but not when treated with trypsin or cholera filtrate (crude neuraminidase). Agglutination of the enzyme-treated sheep erythrocytes is probably due to the unmasking of the receptor site on the surface of erythrocytes (Prokop, *et al.*, 1968).

Johnson (1969, 1970) has extensively investigated the coelomocytes of sea urchins; a similarity of the separating pattern in cellulose acetate-membrane electrophoresis was found between the coelomic fluid and the extracts of coelomocytes, and the results are influenced by the season of collecting the specimens. Further he stated that "vibratile cell" (one of coelomoyctes) diffuses specific macromolecules into the fluid in response to a stress factor. The correlation between coelomic fluid and the extracts of coelomocytes on hemagglutinating activity is obscure.

Hemagglutinins have been demonstrated in several orders of sea urchin (mainly order Camarodonta), and therefore, it would be of interest to compare the hemagglutinins of each species of sea urchin chemically and biologically. At present, further investigations on the chemical properties of the hemagglutinin from *H. pulcherrimus* are in progress.

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SUMMARY

The occurrence of natural hemagglutinins in the coelomic fluid of sea urchins, *Anthocidaris crassispina, Pseudocentrotus depressus*, and *Hemicentrotus pulcherrimus*, was reported.

The coelomic fluid preparations from three species of sea urchin were found to have hemagglutinating activity against rabbit and human erythrocytes but not against mouse and sheep erythrocytes so far examined. Dog erythrocytes were sensitive to the coelomic fluids of *A. crassispina* and *H. pulcherrimus*. The coelomic fluids of *P. depressus* and *H. pulcherrimus* also agglutinated guinea pig erythrocytes.

The hemagglutinins against rabbit erythrocytes from *A. crassispina* and *P. depressus* might be a protein or a protein-like substance and were heat-unstable and resistant to trypsin digestion, but not to bromelain, and were affected by 2-mercaptoethanol though the activity of the former was recovered when 2-mercaptothanol was removed. On the other hand, the hemagglutinin of *H. pulcherrimus* might be a large molecular, complicated carbohydrate, which was heat stable and resistant to trypsin, bromelain, 2-mercaptoethanol, and periodate.

The hemagglutinating activity of the coelomic fluids was greatly enhanced by the addition of calcium but not magnesium, and the hemagglutination by them was not dependent on temperature. Inhibition tests with simple sugars on hemagglutinating activity of the coelomic fluids resulted in failure to determine the specificity of receptor sites for these hemagglutinins. In addition, the results of absorption test on the specificity of the hemagglutinin of sea urchin were discussed.

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LUNAR-DAY VARIATIONS IN SPONTANEOUS ACTIVITY OF THE MONGOLIAN GERBIL[1]

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It is becoming increasingly apparent that organisms possess simultaneously a complex of biorhythms of varying frequencies. A number of small mammals including the white rat, white mouse and the golden hamster have been shown to possess at one and the same time both lunar-day and 24-hour cycles, while also displaying an overt circadian cycle (Brown, Shriner and Ralph, 1956; Brown and Terracini, 1959; Terracini and Brown, 1962; Brown, 1965; Brown and Park, 1967; Boyer, 1970). The 24.8-hour lunar-day cycle appears as the overt cycle in many organisms, particularly those inhabiting the intertidal zone of the ocean (Naylor, 1958; Enright, 1965; Hughes, 1972; Smith and Miller, 1973). In other organisms the lunar-day cycle is of very low amplitude and of a more obscure adaptive value (Terracini and Brown, 1962; Brown, 1965; Brown and Park, 1967; Klinowska, 1970, 1972; Brown and Chow, 1973b).

This paper will investigate whether activity in the Mongolian gerbil, *Meriones unguiculatus,* which has been shown to possess an obvious diurnal cycle (Stutz, 1972) and a more subtle cycle of a synodic monthly period (Stutz, 1973), possesses a lunar-day cycle as well. It is suspected that since a synodic monthly cycle results as a consequence of periodic beats between lunar and solar-day cycles, that a small-amplitude variation of a 24.8-hour period will exist. Should such a cycle be present, its presence would not be particularly adaptive for a desert-dwelling rodent, but more likely a consequence of living in an environment penetrated by subtle pervasive electromagnetic fields having a 24-hour and 50-minute cycle to which the animal is responsive.

MATERIALS AND METHODS

Gerbil spontaneous activity was monitored in small mammal actographs under two lighting regimes—3 foot candles of light from 6 A.M. to 6 P.M. (12–12 LD) and 3 foot candles of continuous light of 3 foot candles intensity (LL). Animals were kept in thermostatically controlled compartments (22 ± 1° C), with 2 animals per compartment. Food and water were present ad libidum and replenished every 5 to 8 days at random hours. Activity was monitored continuously by a 20-channel Esterline Angus Events Recorder and quantified by measuring the percentage of each hour that activity occurred. A scale from 0–10, at intervals of 1, was used, with a value of 10 indicating an animal to be active 100% of an hour.

One group of 6 gerbils was kept in 12–12 LD from May 1, 1968, through August 31, 1968. On September 1, 1968, two of these were removed and used in

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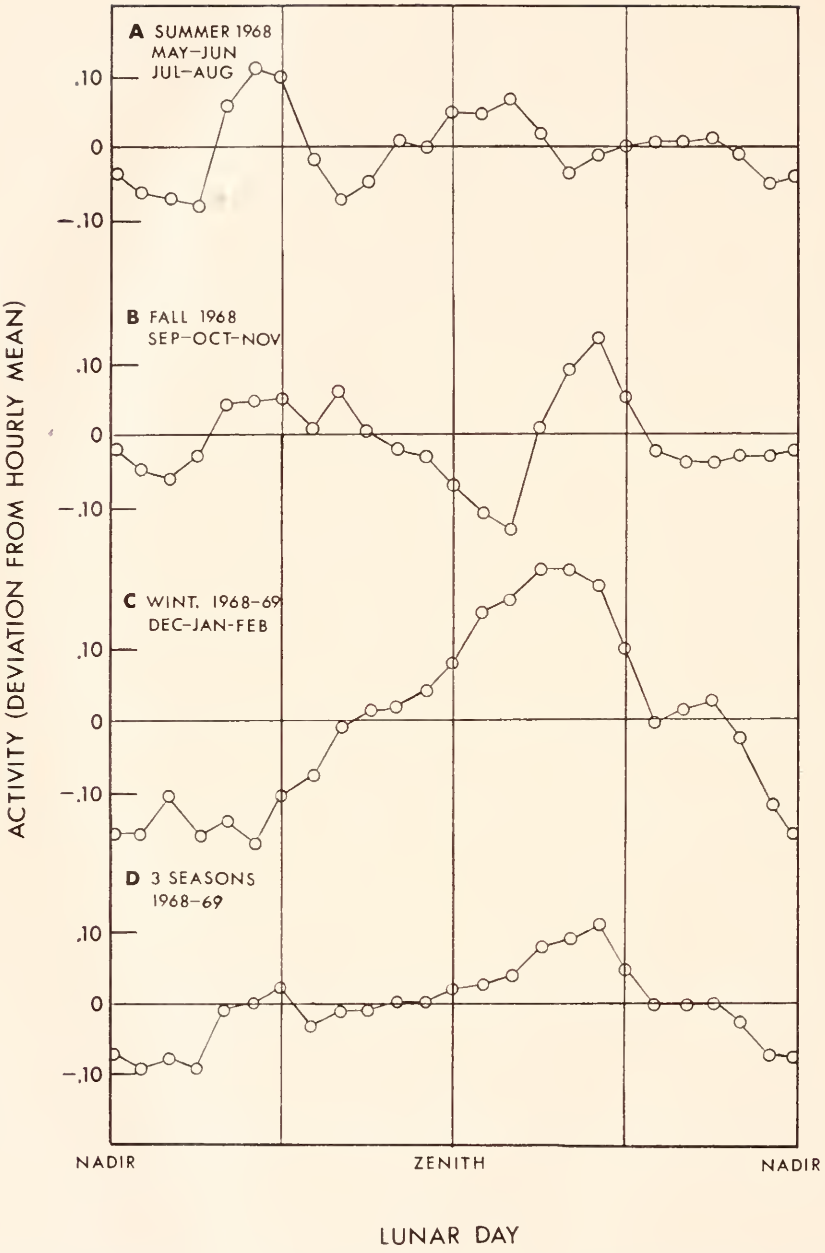

FIGURE 1. Curves A, B, and C represent lunar-day variations in spontaneous activity of gerbils in 12–12 LD for the summer, fall and winter seasons, respectively, in 1968–1969, expressed as deviation from each seasonal hourly mean. Curve D illustrates the mean three-season lunar-day cycle. Values given are three-hour sliding averages.

another experiment. The remaining 4 animals were kept in 12–12 LD through February 28, 1969. A second group of 4 animals was kept in LL from May 1, 1968, through March 15, 1969. Two additional animals which had been in activity cages in the open laboratory from May 1, 1968, were placed in LL on October 1, 1968, and remained in LL through March 15, 1969. Data from one of the animals in LL from May 1 were not used due to a malfunction of the recording apparatus.

An attempt was made to record animals in the two lighting regimes during approximately the same months, as comparisons between these two groups of data are most accurately made when data used cover similar time periods. Brown (1962) has reported an annual rhythm of a lunar-day frequency in fresh water planarians.

Results

12–12 LD

To demonstrate the existence of a lunar-day cycle, data corresponding to approximately the same hour of the lunar day were synchronized by moving successive days of data backwards under earlier days at the rate of 5 hours in 6 days. Since the overt solar-day cycle must be completely randomized, units of 29 days from each calendar month were used, for this permits the solar cycle to scan once per unit the 24 columns of data. This method of analyzing data for lunar-day rhythms has been described by Brown, Freeland and Ralph (1955).

Lunar-day cycles for each of three seasons were obtained by finding the mean amount of spontaneous activity per lunar hour for the season, assigning this seasonal hourly mean a value of zero, and plotting each individual average hourly value for the season as a deviation from this mean. Figure 1A shows the mean daily curve for 6 gerbils from May 1, 1968, to August 31, 1968. These 4 months will be considered the summer season. Figure 1B shows data gathered from 4 gerbils during fall, September 1, 1968, to November 30, 1968. Figure 1C represents similar data from 4 gerbils for the winter days extending from December 1, 1968, to February 28, 1969. The values for Figure 1D were derived in a manner similar to that used for Figures 1A–C, except that the 3-season hourly mean was given the value of zero and the 3-season mean values for each of the 24 hours were expressed as deviations from this mean. The lunar-day cycles in Figures 1A–D are plotted as 3-hour sliding averages.

Figure 1A, the lunar-day cycle for summer, is characterized as a tri-modal curve, with the major maximum at moonrise. A lesser maximum is present at lunar upper transit and extends through the two following hours. The third maximum, of rather low amplitude, occurs shortly after moonset. Minima are present over lunar lower transit, two hours after moonrise, and two hours before the setting of the moon. The range of this cycle is 10%, expressed as per cent of the hourly mean, which was given the value of 100%.

The lunar-day cycle in Figure 1B for the fall season is essentially a bimodal curve, with a maximum extending over moonrise and an even greater maximum, twice as high in amplitude, about one hour before moonset. Minima occur two hours after lunar upper transit and over lunar lower transit. The range of this cycle is 13%.

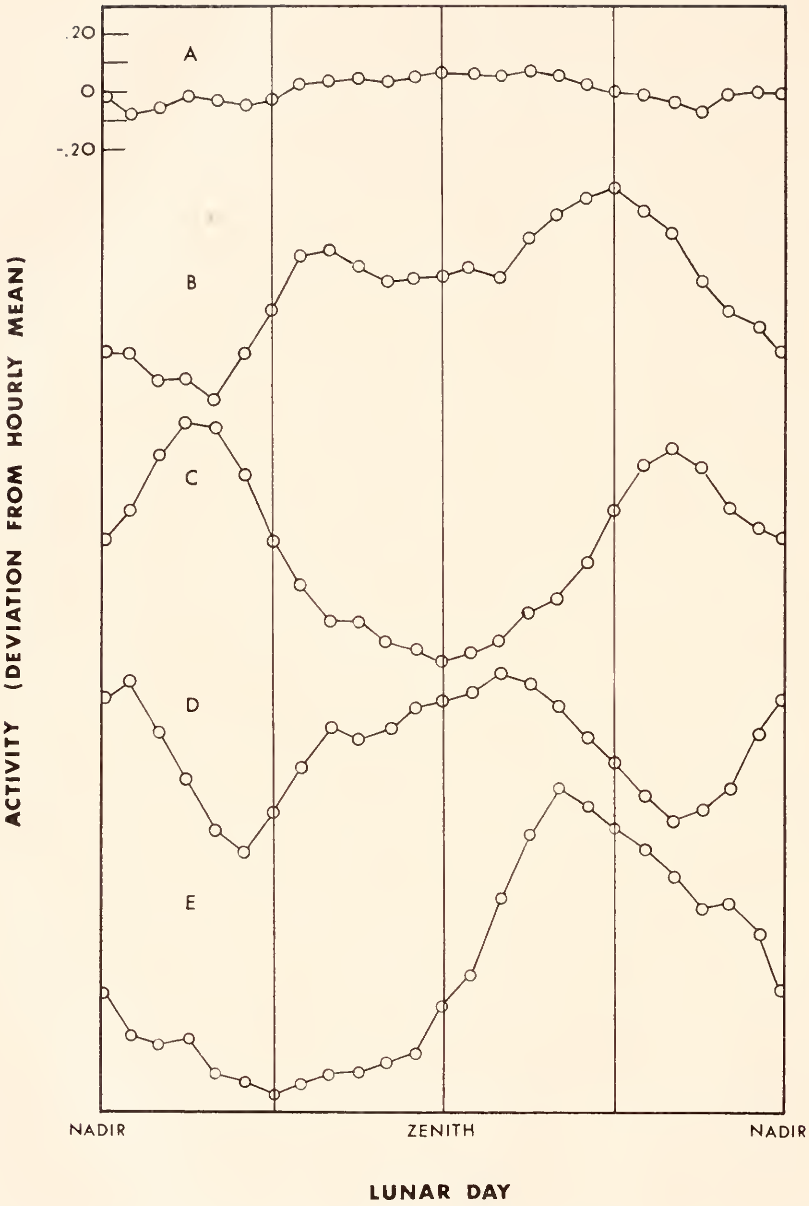

FIGURE 2. Curves A, B, C, D, and E show for gerbils A, B, C, D, and E, respectively, in LL the average lunar-day cycles expressed as deviation from the mean activity per lunar hour for the entire time period used for each animal. Values are three-hour sliding averages.

The lunar-day cycle for the winter season, shown in Figure 1C, is essentially a unimodal curve. The maximum occurs from one to three hours before the setting of the moon, and is almost twice as great in amplitude as maxima occurring in summer and fall. The minimum occurs at lower lunar transit and extends until one hour before moonrise. The range of the cycle for the winter season is 18%.

In Figure 1D, the lunar-day cycle for the combined three seasons is characterized as a bimodal curve. A conspicuous maximum occurs about one hour before moonset, with a subsidiary maximum present at the rising of the moon. A broad minimum is seen at lunar lower transit, while a lesser minimum occurs around lunar upper transit. The range of this three-season cycle is 10%.

When comparing the lunar-day components for the three seasons, it is interesting to note that the form of the summer and fall cycles parallel one another from lunar hours zero through seven, and are very nearly the mirror-image of each other for the remaining hours of the lunar day. The greatest amount of spontaneous activity during the winter season occurs at the same hours of day as it does during the fall. For winter the only minimum, and for summer the lowest minimum occurs over lower lunar transit. The cycle for the fall season also exhibits a broad minimum at this time. The tendency for lunar inversion seems to be great, and maxima and minima appear to be phase-locked to the lunar hours at or close to moonrise, moonset, lunar upper and lower transits.

Constant light (LL)

Data gathered under LL were examined for the existence of a lunar-day cycle in an attempt to verify further the presence of a lunar-day component in the gerbil. Methods of analyzing the data for a lunar-day cycle were similar to those used in the previous section, but in this case both the solar-day cycle and the overt circadian cycle had to be randomized, necessitating period lengths which were simple multiples both of 29.5 days and the period necessary for the circadian cycle to scan the day. Therefore, data for each gerbil were treated as an individual unit of approximately 179 days, and data could not be analyzed for the presence of a seasonal variation.

Figures 2A-E show, for gerbils A, B, C, D, and E, respectively, the lunar-day variation in activity remaining after randomization of overt circadian and solar-day cycles. The following are the period of time (days) used for each animal in this examination: *Gerbil A* (in LL fom May 1, 1968, to March 15, 1969): July 1–December 31, 184 days; *Gerbil B* (in LL from October 1, 1968, to March 15, 1969): October 17–March 15, 149 days; *Gerbil C* (in LL from May 1, 1968, to March 15, 1969): August 1–February 16, 200 days; *Gerbil D* (in LL from October 1, 1968, to March 15, 1969): October 1–February 28, 151 days; *Gerbil E* (in LL from May 1, 1968, to March 15, 1969): August 1–February 28, 212 days. Figure 3 shows the mean cycle for all five gerbils. All values in Figures 2 and 3 are 3-hour sliding averages.

Figures 2A and 2B are very similar, in that times of greatest activity occur when the moon is above the horizon and least activity occurs when the moon is below the horizon. Figure 2A is essentially a unimodal curve of extremely low amplitude, with its maximum at lunar upper transit and minimum around lunar lower transit. Figure 2B may be described further as a bimodal curve with the major minimum around the time of lunar lower transit, and a second minimum at lunar upper transit. Maxima occur around moonrise and moonset. In Figure 2C, a bimodal curve, gerbil activity is greatest essentially when the moon is below the horizon, with maxima occurring close to moonrise and moonset. The least amount

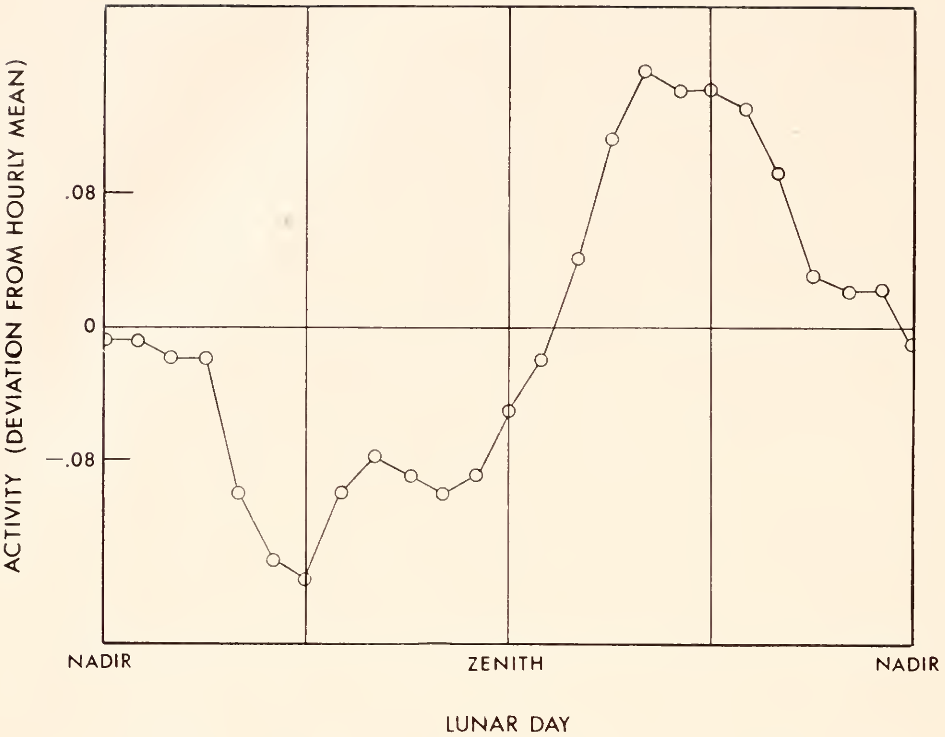

Figure 3. Mean lunar-day cycle for all 5 gerbils—A, B, C, D, and E—in LL. Values are three-hour sliding means.

of activity is during the lunar day, falling to a low at lunar upper transit, with a secondary minimum at lunar lower transit. Figures 2C and 2D are almost perfect mirror images of one another, with gerbil D showing maximum activity when the moon is above the horizon, rising to a peak close to upper transit. A second peak of activity is present at lunar lower transit. Minima occur around times of moonrise and moonset. Figure 2E, a unimodal curve, shows maximum gerbil activity two hours before the setting of the moon and minmum activity at moonrise.

The mean lunar-day cycles for all gerbils seem to have times of maxima and minima locked to the approximate hours of moonrise, moonset, lunar upper and lower transits. There is also an apparent tendency for the cycles to undergo major inversions of one another, and it appears that the cycles are all interrelated with one another in terms of degrees of inversion. These phenomena were also found to be true for those lunar-day activity cycles disclosed in gerbils in 12–12 LD.

Figure 3, the mean lunar-day cycle for gerbils A–E, shows a broad maximum extending over the time of moonset. A secondary, and very low amplitude maximum, is present two hours after moonrise. The major minimum of this cycle is at the time of the rising of the moon.

Expressed as percentages of the mean hourly average, the ranges of the lunar-day cycles in Figure 2 are 11%, 39%, 46%, 29% and 47% for gerbils A, B, C, D, and E, respectively. The range of the mean cycle, Figure 3, for all 5 gerbils is 16%. This range is substantially reduced due to the frequency of cycle inversion found among the 5 gerbils. None of these ranges is insignificant. In fact, the range of 4 out of 5 of the gerbil cycles are quite large compared with those found for lunar-day cycles in other organisms.

Discussion

When appropriate care was used in randomizing the underlying solar and overt circadian rhythms in those gerbils held in LL, a rather large-amplitude average rhythm of a lunar-day frequency was revealed, with maxima close to times of rising and setting of the moon and a conspicuous minimum at lower lunar transit. The lunar-day cycle disclosed in those animals held in 12–12 LD was very similar in form and phase relationship to that of the gerbils in LL.

The phenomenon of inversion of the lunar-day cycle over all or parts of the lunar day was found to exist from season to season in those gerbils in 12–12 LD and also from animal to animal in those gerbils studied individually in LL. Studies of rhythmicity in a variety of organisms have shown that similar periodic inversions of phase, with maintenance of otherwise the same frequency, are common among living things.

Just as underlying solar-day rhythms in the gerbil and a number of other forms appear to be related to one another in cycle form, so also does a similar relationship exist for lunar-day fluctuations. The mean lunar-day cyclic pattern in Figure 1D very closely resembles that of the potato (Brown, 1960), *Fucus* (Brown, Freeland and Ralph, 1955), earthworm (Ralph, 1957), salamander (Brown, Webb, Bennett and Sandeen, 1955), white mouse (Terracini and Brown, 1962), and mealworm (Campbell, 1964). The cycle for the summer season, May through August, is also very similar to that of the golden hamster for the summer lunar month of July–August 1967 (Klinowska, 1972). This similarity, or an inversion of it, throughout all or part of the lunar day, in cyclic pattern between the gerbil and a variety of other species strongly supports the hypothesis that all organisms were influenced by subtle factors of a 24.8-hour frequency penetrating laboratory "constant" conditions.

Recent studies indicate that weak electrostatic fields, gamma radiation and geomagentism, which penetrate easily through buildings and possess the necessary period lengths, may be the timing mechanisms for biological clocks (Brown, 1972; Brown and Chow, 1973a). In addition to their potential role as biological clocks, this family of weak fields has been shown to play other vital roles in the daily lives of organisms (Lindauer and Martin, 1968; Keeton, 1971; Brown, 1971; Wiltschko and Wiltschko, 1972; Rommel and McCleave, 1972; and many others).

Evidence that gerbils respond to the weak horizontal magnetic field or its correlate was shown by Stutz (1971). Unexpected variation in gerbil spontaneous activity from 3–6 P.M. was shown to be correlated (an average of $r = +0.45$; $P = 0.01$; $N = 30$, for each of 6 consecutive months) with the amount of change in horizontal magnetic intensity for the concurrent time period. Significant correlations between the two parameters were not found during any other time periods throughout the day.

The difficulty in imaging a function for a lunar-day component in the terrestrial, desert-dwelling gerbil lends further credibility to the conclusion that the lunar-day periodism in this animal is merely a consequence of existence in a periodic geophysical atmosphere. Brown (1968) hypothesized that the lunar adaptive functions are synchronized to an underlying phase-stable period of a lunar-day frequency in such a manner as to consist of a recycling "tape" which initiates a period of phase-labile activity firmly "coded" to a certain location on the phase-stable tape. In accordance with this hypothesis, it is felt that the gerbil lunar-day phase-stable tape would remain relatively uncoded. Lunar-day periodisms in hamsters (Brown, 1965) and a rat (Brown and Terracini, 1959) have been reported to gain timing control and to hold for an extended time period a behavioral pattern normally a solar-day adaptation. These findings do suggest that the lunar-day phase-stable tape of a land-dwelling species could, however, potentially be coded with a complex pattern of behavior.

SUMMARY

1. Spontaneous activity of male gerbils, *Meriones unguiculatus*, kept in actographs was recorded continuously from May 1, 1968, through March 15, 1969, in Evanston, Illinois. Gerbil activity was studied under two lighting conditions: constant illumination (LL) and 12–12 LD.

2. For those gerbils kept in 12–12 LD a lunar-day periodism of spontaneous activity was disclosed for each of three seasons(summer, fall, and winter). The mean three-season cycle in 12–12 LD exhibited maxima around times of rising and setting of the moon and minima at lunar lower and upper transits.

3. For those 5 animals in LL a lunar-day cycle of activity was reported for each animal over a period averaging 179 days. When the lunar-day cycles for each of the 5 gerbils in LL were averaged together, a mean periodism with cycle form and phase relationship very similar to that of the three-season cycle for gerbils in 12–12 LD was found. The range of this cycle was 16%.

4. All lunar-day cycles studied had maxima and minima locked to the approximate hours of moonrise, moonset, lunar upper and lower transits. There was a strong tendency for lunar inversion, but the cycles appeared to be interrelated with one another in degrees of inversion.

5. These results give support to the hypothesis that the gerbils are responsive to weak geoelectromagnetic fields with period lengths of 24.8 hours from which they are not shielded in the laboratory.

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FEEDING HABITS OF THE SAND SHRIMP *CRANGON SEPTEMSPINOSA*[1]

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The sand shrimp *Crangon septemspinosa* Say is a common estuarine decapod distributed along the northwestern Atlantic from Newfoundland to eastern Florida (Squires, 1965; Williams, 1955, 1965; Price, 1962). Although *C. septemspinosa* is carnivorous (Price, 1962; Regnault, 1970), it also may ingest organic matter in various forms.

Crangon spp. has diverse feeding habits. Lloyd and Yonge (1947) classified *Crangon vulgaris* (= *Crangon crangon*), an European species, as an omnivore after finding algae, polychaetes, gastropods, bivalves, amphipods, fish eggs and fish larvae in the digestive tract. However, they felt that *C. vulgaris* preferred animal tissues. Allen (1960) categorized *Crangon allmani*, another European species, as a carnivore, but noted that sand and mud particles were always found in the stomach. Tiews (1968), in reviewing the literature on *C. crangon*, considered that the species was an omnivore, but animal tissues did comprise the main food items. Kosaka (1970) classified a Japanese species *Crangon affinis* as a carnivore, but sand and mud were always present with the food.

Williams (1955, 1958) found a variety of recognizable and unrecognizable material in the alimentary canal of three species of penaeid shrimp and believed that "softer and more easily digested materials could easily form the bulk of the diet" [1955, page 143]. Dahl (1968) noted that the food of several Australian prawns was small animals and a large amount of unrecognizable material, which might form the main component of the diet. To derive this material, shrimp browse on the microorganisms (bacteria, algae, and microfauna), which grow on the substratum. Frankenberg and Smith (1967) found seven crustaceans that ate fecal material. Also Johannes and Satomi (1966) observed that the grass shrimp *Palaemonetes pugio* assimilated the organic material in and on fecal pellets.

An ability to utilize diverse foods has survival value in a seasonally changing environment. Thus, when preferred food is scarce, organic debris is usually available to estuarine animals (Darnell, 1967, 1968; Odum and de la Cruz, 1967; Odum, 1971).

The objective of this study was to determine the kinds of food utilized by *C. septemspinosa*. Stomach contents and time of feeding were observed in a natural population: rate of digestion and growth performance on prepared diets were evaluated in the laboratory.

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MATERIALS AND METHODS

Collecting area

Shrimp were seined (2 × 1 m; 2 mm mesh) from sand flats in the Pettaquamscutt River, Rhode Island, near Sprague Bridge (41° 26′ 55″ N, 71° 27′ 05″ W). This river empties into Rhode Island Sound near the mouth of Narragansett Bay. The station had the following characteristics: water depths of 0.5–1.0 m, average salinity of 27‰, fine to coarse sand, and current velocities of approximately 20–30 cm/sec at full tidal flow.

Stomach analyses

From May to October, 1971, a total of 225 stomachs were examined. Shrimp were collected during the day; those selected had the anterior chamber of the proventriculus expanded with material, which could be seen through the carapace. The shrimp were frozen immediately; after thawing the carapace was lifted and the internal organs exposed. The stomach was removed and opened on a microscope slide. The contents were examined with a dissecting microscope (9–75 ×) and identified. Frequency of occurrence was expressed as the number of stomachs in which each food item occurred as a percentage of the total number of individuals examined. Volumetric importance of each food type was made by visual estimate.

Time of feeding

Sampling was conducted over a 24-hour period on July 7 and 8, 1971. Each hour, 30 shrimp were selected randomly from seining and frozen immediately. Later estimates of stomach fullness were made (full, half-full, and empty). The contents of full and half-full stomachs were examined for trituration.

Rate of digestion studies

Shrimp, 15 to 54 mm in length, were collected on July 20, 1971 and were separated into 5 mm size groups. Three shrimp from each group were placed in a plastic container (20 × 20 × 40 cm) with holes drilled in the sides for water circulation. The vessels, which contained 1–2 cm of clean sand, were covered and submerged in a water table (15 cm water depth).

Seawater was filtered through a sand bed (30 × 50 × 30 cm) to remove particulate material before flowing through the water table. The filter retained approximately 93% of the chlorophyll *a* in the entering water (chlorophyll determined by a method of Strickland and Parsons, 1965). The flow rate through each filter was approximately 550 ml/min. Each water table had two filters.

Three diets of varying texture were tried: tissues of *Crangon*, fish meal (Point Judith Fisherman's Cooperative, Point Judith, Rhode Island), and the clam *Mercenaria mercenaria*. All tissues were dehydrated, and 6 g of each diet were mixed with 0.5 g of carmine and 30 ml of 2.2% agar (Bacto-agar, Difco Laboratories, Detroit, Michigan).

Before each trial, shrimp were starved for two days. Blocks of food were then placed in each compartment; and upon ingestion, the dyed food was easily seen through the carapace. During the first 6 hours, the shrimp were observed hourly for reduction of coloration in the stomach and for its appearance in the intestine. One shrimp from each of the three feeding groups was sacrificed each hour over the 6-hour observational period, and estimates of the rate and degree of physical digestion of its stomach contents were made. Inspections were also made at 12 and 24 hours after feeding.

Feeding studies

All procedures were as described previously, except that the experiments were 8-weeks long. Growth was estimated from successive measurements (to the nearest mm) on the same individual every 2 weeks.

The following diets were tested: *Candida sake,* a marine yeast isolated from macrovegetation taken from tide pools at Narragansett, Rhode Island and grown in malt extract broth (25 g/l of seawater); Baker's yeast (Fleishmann's Yeast, Standard Brands, Inc., New York); brine shrimp (Metaframe Co., San Francisco, California); an unknown bacterium, probably a member of the *Pseudomonas* group isolated from Narragansett Bay seawater and grown in an "OZR" medium (1.0 g yeast extract and 1.0 g trypticase/l of seawater); calanoid copepods taken over a year's period in Narragansett Bay; *Spartina alteriflora* detritus and attached microflora collected at Bissels Cove in North Kingston, Rhode Island (the size fraction between 0.500 and 0.017 mm); TetraMin, a tropical fish food (TetraKrafteWerke, West Germany); finely chopped hard-boiled egg; and glycogen obtained from Nutritional Biochemical Co., Cleveland, Ohio. All other foods were as described previously.

Foods were dehydrated by heat or freeze-drying. Six gram portions were mixed with 30 ml of 2.2% agar; the gel was cut into 2 g blocks, which were fed to the shrimp daily.

Experiments were run during spring, summer, late summer, and fall; mean water temperatures were 15, 20, 20, and 17° C respectively. Controls were given fresh clam tissue. A second control was maintained without food during the summer and late summer experiments.

The growth rates were calculated according to the following equation which permits comparisons among groups of growth curves but makes no assumptions about the growth model (Rao, 1958):

$$B = \sum g_i X_i$$

where B = growth rate in arbitary units; g_i = the sum of the growth increments in mm among all treatments for each growth period; X_i = the size increment for each shrimp during each growth period; and i = the number of incremental periods. To equalize the variances, the growth rates were transformed by square root.

Treatments within each season were compared by one-way analysis of variance (Ostle, 1963). Fisher's Least Significant Difference (LSD) was used for pairwise comparison of groups with multiple means (Fryer, 1966). If two means differed by the LSD calculated at a preselected probability, they were statistically different.

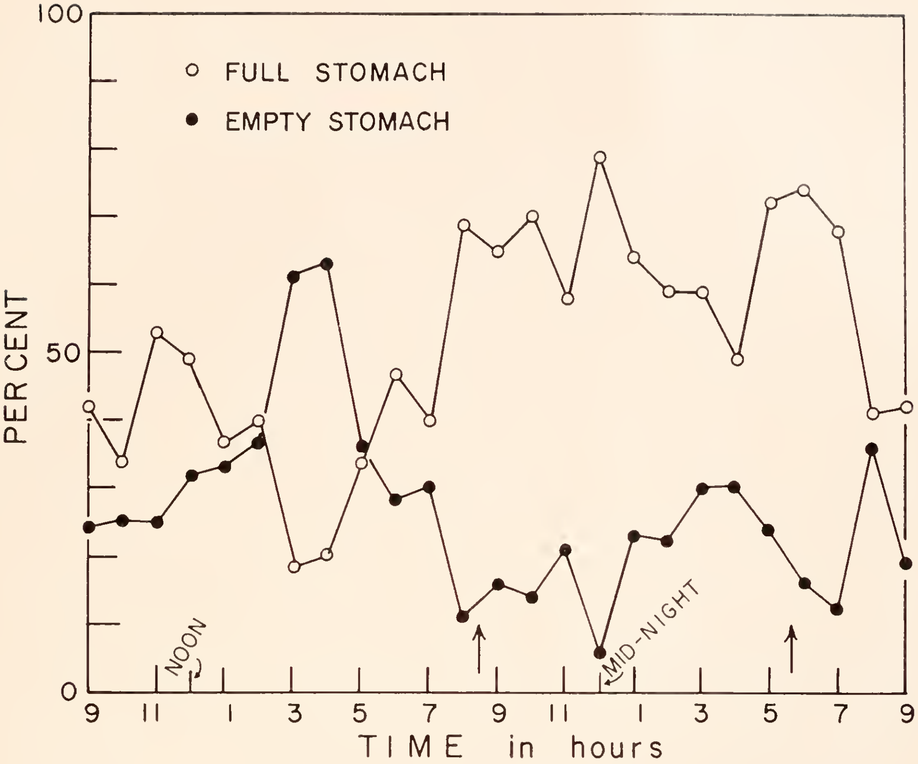

FIGURE 1. Stomach fullness compared with time of day for a field population of *C. septemspinosa*. The arrows indicate the time of sunset and sunrise.

RESULTS

Stomach analyses

In terms of volume and frequency of occurrence, organic debris, sand, and crustacean parts were the major components of 225 stomachs examined. Organic debris comprised 85% of the volume and occurred in all the stomachs examined. Sand had a frequency of occurrence of 76% and comprised approximately 4% of the total volume. Crustaceans parts occurred in 57% of the stomachs examined and accounted for 8% of the total volume. Minor food items (< 1% frequency of occurrence) included: copepods, plant material, polychaete fragments, amphipods, mollusks, fecal pellets, fish scales, nematodes, ostracods, and eggs from invertebrates. Seasonal trends in the proportions of dietary components were not apparent.

Time of feeding

Based on stomach fullness, the major feeding period was at night (Fig. 1). The onset of feeding seemed to be initiated at darkness (sunset on July 7 was 8:32 DST), and feeding activity peaked once at midnight and again approximately at dawn (sunrise on July 8 was 5:40 DST).

TABLE I

Mean growth rates ($\bar{x}$ ± sd) of C. septemspinosa *fed the respective diets during four seasons. The growth rates, B, may be compared within seasons, but should not be compared between seasons because of data transformations. Note the mean water temperature for each season. All dried foods were embedded in an agar binder. See the text for the source and preparation of the foods and for an explanation of Categories I–IV. Fisher's LSD was calculated at* P = *0.05; N = 12 for each determination except fall, where N = 23.*

Food	Spring 15° C	Growth rate B		Fall 17° C
		Summer 20° C	Late Summer 20° C	
Category I				
Fresh *Mercenaria*	14.18 ± 3.31	13.02 ± 3.54	21.38 ± 5.17	
Frozen *Artemia*			21.95 ± 4.31	30.48 ± 5.63
Hard-boiled egg				28.34 ± 5.37
Category II				
Dried fish meal	10.94 ± 2.47			28.34 ± 6.01
Freeze-dried *Crangon*	10.87 ± 3.19		17.82 ± 6.97	
Freeze-dried *Mercenaria*	10.06 ± 1.93			
Freeze-dried copepods		8.76 ± 2.05		
Freeze-dried *Artemia*			15.06 ± 4.14	26.01 ± 3.20
Category III				
Freeze-dried beef liver	9.37 ± 2.94			
Freeze-dried marine yeast	9.24 ± 2.68			
Agar + glycogen (0.5 g)	8.92 ± 2.43			
TetraMin (dried)		7.09 ± 1.96		
Baker's yeast (dried)		7.65 ± 2.80		
Freeze-dried *Spartina* detritus		6.42 ± 1.49		
Freeze-dried bacteria			9.77 ± 2.91	
Frozen bacteria				20.74 ± 3.73
Category IV				
Starved	4.61 ± 4.23	2.83 ± 3.01	5.40 ± 5.21	7.68 ± 6.67
LSD	2.42	2.11	4.06	3.05

Shrimp with full or half-full stomachs were examined to estimate the degree of trituration. Food retained its identity for approximately 2 hours after sunset.

Underwater observations in nature showed that *C. septemspinosa* fed on fresh animal tissue. During the day, *Crangon* were immediately attracted to shucked mussel (*Mytilus edulis*) and would ingest the tissue within two minutes.

Rate of digestion studies

A few minutes after the ingestion of dyed food, the cardiac stomach and hepatopancreas became bright red. Within an hour of ingestion, food and dye reached the intestine, and production of fecal pellets began shortly thereafter. Coloration faded over time, and by 6 hours the hepatopancreas was pale pink. The color did not diminish further because the dye was apparently incorporated into the tissue of the digestive diverticula.

Stomach contents were examined 1 hour after feeding. Paste-like consistency indicated efficient trituration by the gastric mill. The cardiac portion of the stomach was empty 6–12 hours after feeding.

Feeding studies

Comparisons within seasons (Table I) showed the growth rates differed (analysis of variance, $P = 0.01$; Fisher's LSD, $P = 0.05$) between treatment means. Because of data transformations, mean growth rates should not be compared between seasons.

Foods may be classified into four categories based on origin and processing: Category I—fresh or frozen animal tissues generally of marine origin (*Mercenaria*, *Artemia*, and hard-boiled egg); Category II—dried animal tissues of marine origin (*Crangon*, fish meal, *Mercenaria*, copepods, and *Artemia*); Category III—dried animal and microbial tissues (beef liver, marine yeast, agar, TetraMin, Baker's yeast, *Spartina* detritus, bacteria); Category IV—starved. To rule out seasonal influences, the growth increments within each of the four categories were pooled and new growth rates were calculated. Within each category, most treatments were found to produce equal growth rates (analysis of variance; $P = 0.05$). Mortalities for the 8-week period were: Category I—10%; Category II—30%; Category III—40%; Category IV—55%.

Starved groups were used for only the summer and late summer groups, but the results were extrapolated to spring and fall. An assumption of this extrapolation was that metabolism of starved shrimp would be maximal when the water temperature was the warmest (the summer months) and that the rates would be lower at cooler water temperatures.

Since there were always significant differences in growth rates between the starved groups (Category IV) and the others, *C. septemspinosa* derived some nutritional value from all the foods that were offered. The shrimp grew best when eating fresh animal tissues generally of marine origin (Category I), but to a lesser extent, they utilized and grew on other organic material.

DISCUSSION

Organic debris was a major component in the field diet of *C. septemspinosa*. It had several origins because detritus as well as tissues made undistinguishable by trituration were included in this category. Sand in the diet probably contributed adsorbed organic compounds and associated bacteria, which could serve as nutrients (Odum, 1971), but sand could also assist in trituration (Tiews, 1968).

Since the data for stomach analyses were taken during the daylight hours and the shrimp generally fed at night, it was possible that trituration made identification of food items difficult. The shrimp might have ingested anything of caloric value that was encountered, but it would be rapidly and efficiently reduced to organic debris.

Based on results similar to our data, Price (1962) classified *C. septemspinosa* as a secondary consumer. He found that organic debris comprised approximately 60% of the material ingested; each of nine other groups contributed less than 10%. Various sources of crustaceans (involving five groups) accounted for 24% of the volume. Our data showed that crustaceans (crustacean parts and copepods) comprised only 9% of the volume. The difference might be due to the uncertainty of estimating volumes or the difference may be attributed to crustacean abundances between Delaware and Rhode Island. Price (1962) also noted variations in the stomach contents over a 7-month period. Such variation may have arisen from availability and not preference for specific foods.

Crangon septemspinosa, *C. crangon*, *C. allmani*, and *C. affinis* are carnivores (Lloyd and Yonge, 1947; Allen, 1960; Price, 1962; Tiews, 1968; Kosaka, 1970; Regnault, 1970). However, a wide variety of materials found in the digestive tracts (*e.g.*, mud, sand, algae, detritus) suggests that *Crangon* spp. ingest anything encountered. The identity of many items is quickly destroyed by trituration. The resistance of chitin, shells, and setae to digestion and slow passage of these items through the digestive tract may bias the results towards carnivoristic feeding. The large amount of unidentifiable organic detritus found by Price (1962) and our study suggest that detritus is a dietary constituent.

Much of the organic debris noted in the stomachs of the shrimp may be of plant origin. Generally this cellulose is of limited nutritional or caloric value because of degradation by bacterial and leaching processes (Nykvist, 1959; Darnell, 1967; Odum and de la Cruz, 1967; Kormondy, 1968; Udell, Zanudsky, and Dohery, 1969). The nutritional value of this detrital material is derived from the extensive microflora (protozoa, bacteria, yeast) that colonizes the surface (Russell-Hunter, 1970; Odum, 1971).

Organic matter may also be derived from the aufwuchs community that develops on macrovegetation (Brown, 1962). Appendages and mouth parts of the shrimp are modified for collecting and ingesting small particles such as benthic diatoms. Diatom frustules and aufwuchs were not found in the stomachs of *C. septemspinosa*, so the ecological significance of this material is uncertain.

The anterior chamber of the proventriculus of *Crangon* acts as a crop for food that is cut and masticated by the mandibles or for food that is small enough to be gulped (Dahl, 1968; Tiews, 1968). The gastric mill reduces the food to a paste where it then passes into the digestive diverticula of the hepatopancreas for digestion or onto the mid-gut for osmotic regulation and defecation. Dahl (1968) noted similar rates for ingestion, passage of food to the hepatopancreas, and defecation as we observed with *C. septemspinosa*.

The incorporation of the carmine ($< 5\ \mu$ diameter) into the tissues of the hepatopancreas was in direct contrast to the work of Forster and Gabbott (1971). They noticed no uptake of chromic oxide by the digestive diverticula of the prawns *Palaemon serratus* and *Pandalus platyceros*. The efficient action of the pyloric filter in the prawns apparently prevented the chromic oxide from passing into the diverticula of the hepatopancreas. In contrast, *Crangon* does have a gastric mill and presumably does not need as an efficient pyloric filter, which would permit some of the carmine to enter and stain the hepatopancreas.

Our data show that a variety of foods are of nutritive value to *C. septemspinosa*. Foods that promoted the best growth in the laboratory (Category I) are the preferred foods in nature. However, results demonstrate that representative microflora associated with *Spartina* detritus supply nutrients to *C. septemspinosa*.

Forster and Gabbott (1971) showed that prawns assimilate foodstuff of animal and vegetable origins. Bärlocher and Kendrick (1973) found that the amphipod *Gammarus pseudolimnaeus* utilizes the fungal populations on leaf litter. Sick, Andrews, and White (1972) demonstrated that semipurified diets provide nutrients to penaeid shrimp much better than natural foods. Decapods have a full complement of digestive enzymes (*i.e.,* proteases, lipases, and carbohydrases; Yonge, 1924; Vonk, 1960; Dahl, 1968) allowing the organism to digest and utilize the bulk of the material ingested. Therefore, the limitation of feeding may not be due to digestibility, but with supplying the proper nutrients (*i.e.*, having a small C:N ratio; Russell-Hunter, 1970) and with the olfactory stimuli to find and ingest the food (Barber, 1961).

Olfactory quality could account for the different rates of growth between food Categories I, II, and III (Table I). Drying, either by heat or freeze-drying, apparently removed enough of the chemical stimuli to reduce the olfactory quality (see Wilcox, 1972). The difference was most obvious between foods of Category I and II, where the difference in growth may be attributed to drying.

The reduced growth rates in Category II and III had two possible causes: olfaction and nutrition. Foods of non-marine origin (*e.g.,* Baker's yeast) could be lacking the olfactory chemicals that evoked feeding responses. Also these foods could be lacking or low in essential amino acids, lipids, or other nutrients required by the shrimp. For example, frozen bacteria were avidly consumed, whereas the freeze-dried bacteria was ingested to a lesser extent; the difference was probably due to olfactory stimuli. For the freeze-dried and frozen bacteria, no difference was observed in the growth rates (Table I, Category III), which seemed to indicate that the shrimp were not receiving a nutritionally balanced food. In contrast, when hard-boiled egg was fed to the shrimp, growth was equal to that realized by shrimp fed foods of marine origin.

Observations on the behavior of *C. septemspinosa* show that it follows a zigzag path as the shrimp homes on the scent of food (Wilcox, 1972). Similar types of homing behavior are also common for other crustaceans (Pardi and Papi, 1961). The chemical stimulus for feeding appears to be general, because non-marine substances (*e.g.*, Baker's yeast, hard-boiled egg) evoke the response (Wilcox, 1972). Foods that are preserved by drying, however, are often not ingested. Heat or freeze-drying apparently modifies or removes water-soluble compounds that are presumably responsible for initiating the feeding response. The unsuitability of dried foods, then, is not necessarily in the nutritional quality of the food, but in the ability of the shrimp to locate it. Interesting areas for future research are the separation and understanding of olfactory and nutritional qualities of food.

For a complete feeding cycle, an organism must be stimulated by olfaction to seek the food, the food must satisfy the taste and tactile receptors, and, finally, feeding stops when the stomach is full. At any stage the organism may reject a food item. Preferences may exist, and the animal may seek these foods first, but if these preferred components are scarce in the ecosystem and the animal is hungry, it will ingest a variety of substances until satisfied. The term omnivore is aptly applied to *C. septemspinosa.*

Nutritional requirements of arthropods are known to be specific (Provasoli, Shiraishi, and Lance, 1959; Provasoli and Shiraishi, 1959; Provasoli and D'Agostino, 1962, 1969; Takano, 1967; Subrahmanyam and Oppenheimer, 1969). Poor growth in several of our experiments may be due to nutritional deficiences of the food. Regnault (1970) has demonstrated the importance of a proper diet for optimum growth of juvenile *C. septemspinosa*.

Welsh (1970) determined that *C. septemspinosa* was active at night. Hagerman (1970) found that the locomotory activity of *C. vulgaris* began at darkness and observed 2–3 periods of peak activity during 16 hours of darkness. These results agreed with 2 periods of nighttime feeding activity we observed (Fig. 1). Our data also indicate that *C. septemspinosa* is an opportunistic feeder. They feed predominantly at night, but they will take preferred foods during daylight hours.

Haefner (1969a, 1969b, 1970, 1971, 1972) and Huddart and Arthur (1971) showed how *Crangon* spp. respond and adapt to physical and chemical variations encountered in an estuary. Unspecialized feeding is also believed to be another adaptation by *C. septemspinosa* to cope with the vagaries of food in the estuary.

Summary

Laboratory studies were conducted to compare growth rates of the estuarine sand shrimp *Crangon septemspinosa* on various natural and artificial diets. Field studies examined the types of food consumed by the shrimp.

In nature, 85% of the material in the stomach is organic debris; sand, crustacean parts, copepods, plant material and polychaetes make up the remainder. Some of the organic debris results from trituration of ingested tissues, but an undetermined portion is ingested as detritus.

In the laboratory, shrimp grow rapidly on diets of *Artemia salina, Mercenaria mercenaria* and hard-boiled egg. Fish meal, copepods, beef liver, tropical fish food, agar enriched with glycogen, bacteria, and *Spartina alterniflora* detritus with associated microflora are also utilized, but growth is retarded.

Crangon septemspinosa utilizes a variety of foods. The shrimp have a preference for animal tissues of marine origin and grow best on these foods, but they have the ability to utilize food of microbial and terrestrial origins.

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